



PHD

Analytical and chromatographic studies on aminoglycoside antibiotics.

Lacy, Jonathan Ernest

Award date:
1984

Awarding institution:
University of Bath

[Link to publication](#)

Alternative formats

If you require this document in an alternative format, please contact:
openaccess@bath.ac.uk

Copyright of this thesis rests with the author. Access is subject to the above licence, if given. If no licence is specified above, original content in this thesis is licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC-ND 4.0) Licence (<https://creativecommons.org/licenses/by-nc-nd/4.0/>). Any third-party copyright material present remains the property of its respective owner(s) and is licensed under its existing terms.

Take down policy

If you consider content within Bath's Research Portal to be in breach of UK law, please contact: openaccess@bath.ac.uk with the details. Your claim will be investigated and, where appropriate, the item will be removed from public view as soon as possible.

ANALYTICAL AND CHROMATOGRAPHIC STUDIES
ON AMINOGLYCOSIDE ANTIBIOTICS

Submitted by Jonathan Ernest Lacy
for the degree of Ph.D.
of the University of Bath
1984

COPYRIGHT

"Attention is drawn to the fact that copyright of this thesis rests with its author. This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author".

RESTRICTIONS ON USE

The thesis may be made available for consultation within the University Library and may be photocopied or lent to other Libraries for the purpose of consultation.

J. E. Lacy

ProQuest Number: U341572

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



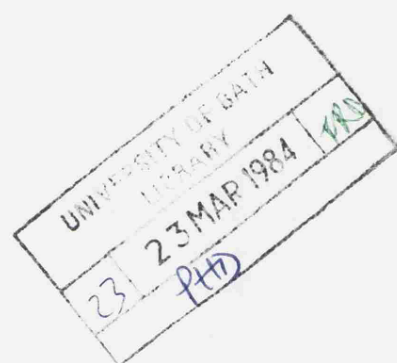
ProQuest U341572

Published by ProQuest LLC(2015). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code.
Microform Edition © ProQuest LLC.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346



ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. M.G. Rowan for his excellent supervision and for many informative and fruitful discussions. I am also indebted to Prof. R.T. Parfitt for his kind assistance and for providing the gentamicin commercial samples. I would also like to record my appreciation of the invaluable help of Mr. R. Sadler and other technical staff of the School of Pharmacy and Pharmacology.

I wish to thank Dr. D. Scott of the General Hospital, Birmingham, for providing patient plasma samples and comparative microbiological assay results, as well as to Mr. M. Rossiter of the Department of Chemistry, University College, Cardiff for running numerous compounds by Field Desorption Mass Spectrometry.

Finally, I would most especially like to thank Helen, my wife, for her tremendous help and patience, in spite of my preoccupation with this research.

SUMMARY

Minor components of the gentamicin series were isolated from commercial samples of gentamicin sulphate. Mass spectrometry and nuclear magnetic resonance (NMR) were applied to elucidate structures. Total identification was not achieved for all components though evidence for novel central sub-units was presented. An unusual site for N-methylation was discovered at the 3-position on gentamicin C₂.

Reversed phase high performance chromatography (HPLC) with pre-column derivatisation was used to quantitate gentamicin component levels in commercial samples from various sources. A method for the measurement of gentamicin in blood plasma by HPLC was developed. Methods for extraction of gentamicin from plasma constituents were evaluated and a chosen method used to determine levels in over fifty patient samples. These values were compared with those found by microbiological assay and a close relationship was observed. Pharmacokinetic parameters were estimated for the individual gentamicin components (C₁, C_{1a} and C₂). Gentamicin C_{1a} was found to possess a longer plasma half life than the other two components.

The fluorometric reaction of ortho-phthalaldehyde (OPA) with the gentamicin components was studied using ¹H and ¹³C NMR. The reactive intermediate was shown to be a dihydro-isobenzofuran and a reaction pathway proposed. The OPA reagent was found to react with all the primary amino functions on the

gentamicin components. Ninhydrin was used to confirm this result.

The retention characteristics of the gentamicin-OPA derivatives in the HPLC system used were investigated and an explanation of the factors governing the observed behaviour, proposed.

Differences in the order of elution of the components, reported

. in the literature, were also explained.

CONTENTS

	<u>Page</u>
ACKNOWLEDGEMENTS	i
SUMMARY	ii
CHAPTER 1. INTRODUCTION	1
CHAPTER 2. ISOLATION AND IDENTIFICATION OF MINOR COMPONENTS OF THE GENTAMICIN SERIES	35
2.1 Introduction	35
2.2 Materials and Methods	
2.2.1 Materials	38
2.2.2 Methods	39
2.3 Results and Discussion	45
2.3.1 HPLC Semi-Preparative Isolation of Minor Components	45
2.3.2 Spectral Characteristics of the Gentamicin C Components	46
2.3.3 Isolation of Minor Components	61
2.3.4 Spectroscopic Study of Netilmicin	86
CHAPTER 3. QUANTITATION OF COMPONENT LEVELS OF GENTAMICIN IN COMMERCIAL SAMPLES OF GENTAMICIN SULPHATE AND IN BLOOD PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY	95
3.1 Introduction	95
3.2 Materials and Methods	98
3.2.1 Materials	98
3.2.2 Methods	99
3.2.2a General HPLC Method	99
3.2.2b Gentamicin component ratio	

	<u>Page</u>
determinations in commercial material samples and pharma- ceutical preparations	100
3.3.2.c Assay of Gentamicin levels in blood plasma	103
3.3 Results and Discussion	106
3.3.1 Determination of Component Levels in Commercial Material	106
3.3.2 High-performance Liquid Chromatographic Analysis of Gentamicin in Blood Plasma	127
CHAPTER 4. ORTHO PHTHALALDEHYDE REACTION IN RELATION TO GENTAMICIN	157
4.1 Introduction	157
4.2 Materials and Methods	
4.2.1 Materials	163
4.2.2 Methods	163
4.2.2.a Nuclear Magnetic Resonance Study of the Reaction	163
4.2.2.b Fluorescence measurement of Gentamicin-OPA reaction	167
4.2.2.c Ninhydrin reaction with Gentamicin	167
4.3 Results and Discussion	170
4.3.1 Nuclear Magnetic Resonance Study of the Reaction Mechanism between o- phthalaldehyde and Primary Amines	170

	<u>Page</u>
4.3.1.a NMR of Individual Reactants	170
4.3.1.b NMR of o-Phthalaldehyde	
Reagent	175
4.3.1.c o-Phthalaldehyde Reaction with	
Standard Amines	195
4.3.1.d Quantification of extent of	
OPA Reaction by NMR	206
4.3.2 Fluorescence Measurement of o-Phthal-	
aldehyde Reaction	212
4.3.3. Combined Ninhydrin/o-Phthalaldehyde	
Reaction with Gentamicin	214
CHAPTER 5. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC	
RETENTION CHARACTERISTICS OF GENTAMICIN-	
ORTHO-PHTHALALDEHYDE DERIVATIVES	220
5.1 Introduction	220
5.2 Materials and Methods	228
5.2.1 Materials	228
5.2.2 Methods	229
5.2.2.a Effect of Temperature on Retention	229
5.2.2.b Effect of pH on Retention	230
5.2.2.c Addition of Salts to the Mobile	
Phase	230
5.3 Results and Discussion	231
5.3.1 Effect of Temperature on Retention	231
5.3.2 Effect of pH on Retention	235
5.3.3 Effect of Salt on Retention	242
Literature Cited	284

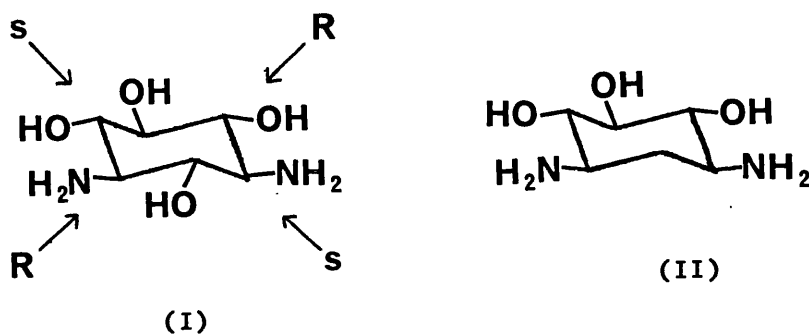
CHAPTER 1

INTRODUCTION

The aminoglycoside aminocyclitols are a group of antibiotics which are still clinically very important^{1,2}. The first member, Streptomycin, was isolated from *Streptomyces griseus* in 1944³. Thereafter other structurally related antibiotics such as the neomycins⁴, the kanamycins⁵, paramomycins⁶ and lividomycins^{7,8} were isolated from other *Streptomyces* species after an intensive search of cultures of organisms similar to fungi. In 1963 gentamicin, a complex of three components, was discovered in two species of *Micromonospora*^{9,10}; *M. purpurea* (N.R.R.L. 2953) and *M. echinospora* (N.R.R.L. 2985). It was the first antibiotic to be found in this genus and subsequent investigation led to the isolation of the sisomicins from *M. inyoensis* in 1970¹¹. Both of these genera, *Micromonospora* and *Streptomyces*, belong to a group of organisms called the Actinomycetes¹² which are thought to be related to gram positive bacteria¹³. Tobramycin, one of the most recent natural aminoglycosides in clinical use, was discovered in 1967¹⁴ as nebramycin factor 6 from *S. tenebrarius*¹⁵. Sagamicin, introduced into clinical practice in Japan recently, has been identified as gentamicin C_{2b}¹⁶. Research has also been directed towards developing semi-synthetic aminoglycosides. Amikacin¹⁷ and Butakacin¹⁸ have both been derived from Kanamycin. A, whilst Dibekacin¹⁹ is from Kanamycin B. A more recent synthesis was Netilmicin from Sisomicin²⁰.

Chemistry of the Aminoglycosides

The aminoglycoside family of antibiotics is characterised by an aminocyclitol subunit; either a streptamine (I) or a 2-deoxy-streptamine(II) ring^{1,21,22}.



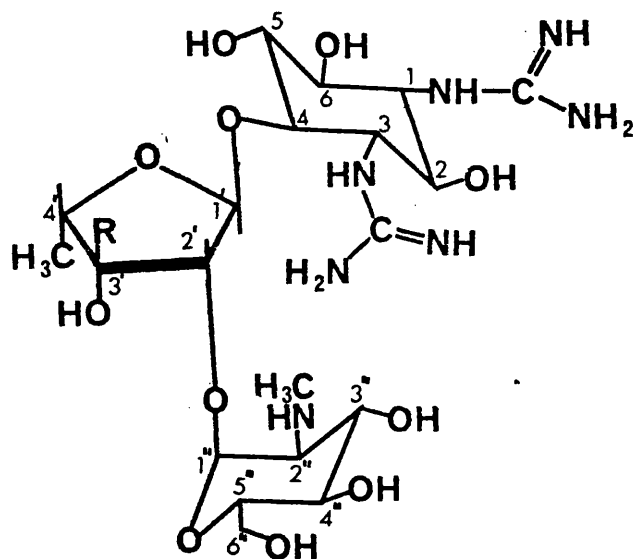
The streptamine ring was isolated from Streptomycin²³ as a N^1N^3 -diamidino derivative. Neomycin was the first antibiotic found to contain the deoxystreptamine^{24,25} moiety which was isolated by acid hydrolysis. Since then most aminoglycosides discovered have had (II) as part of the structure. The configuration of 2-deoxystreptamine was found to be all trans by the size of the coupling constants measured by Nuclear Magnetic Resonance (N.M.R.)²⁶. The deoxystreptamine molecule is seen to possess two pairs of enantiotropic centres with the numbering starting at the nitrogen bearing carbon which has the R-configuration²². The aminoglycosides can be further subdivided by the position of attachment of the two glycosidic bonds to the 2-deoxystreptamine. It is known that the neomycins, lividomycins and paramomycins are 4,5 substituted

whilst the kanamycins and gentamicins are 4,6 substituted. The amino-sugar attached to the 4-position receives primed numbers whilst that attached to the 5 or 6-carbon receives double primed numbers.

Streptomycin has the structure illustrated in Figure 1.1A with a streptidine ring linked to L-methylglucosamine by a sugar moiety called streptose. Dihydrostreptomycin has the reduced streptose ring present as shown in Figure 1.1B.

Neomycin is a complex of three major components as shown in Figure 1.2^{1,26,27}. Neomycin B is the major component and differs from Neomycin C only in the stereochemistry of the aminomethyl group attached to C-5'''. Neamine is a degradation product of Neomycins B and C as well as a fermentation product²⁵. Other minor components have been found to be co-produced but they possess little biological activity^{28,29}. In the United Kingdom a limit of 2% w/v of neamine in neomycin sulphate is set but there is no limit on the proportion of neomycin C present³⁰. However, Framycetin is classified as being neomycin B and limits of 3% w/v neomycin C and 1% w/v neamine are defined³⁰. Neomycin C present is measured by ion-exclusion chromatography with ninhydrin monitoring whilst the neamine level is controlled by Thin-Layer Chromatography (TLC) using a reference standard. The FDA in the United States requires only a potency determination and no assessment of component composition³¹. This is because neomycin is not administered parenterally, and so less strict limits

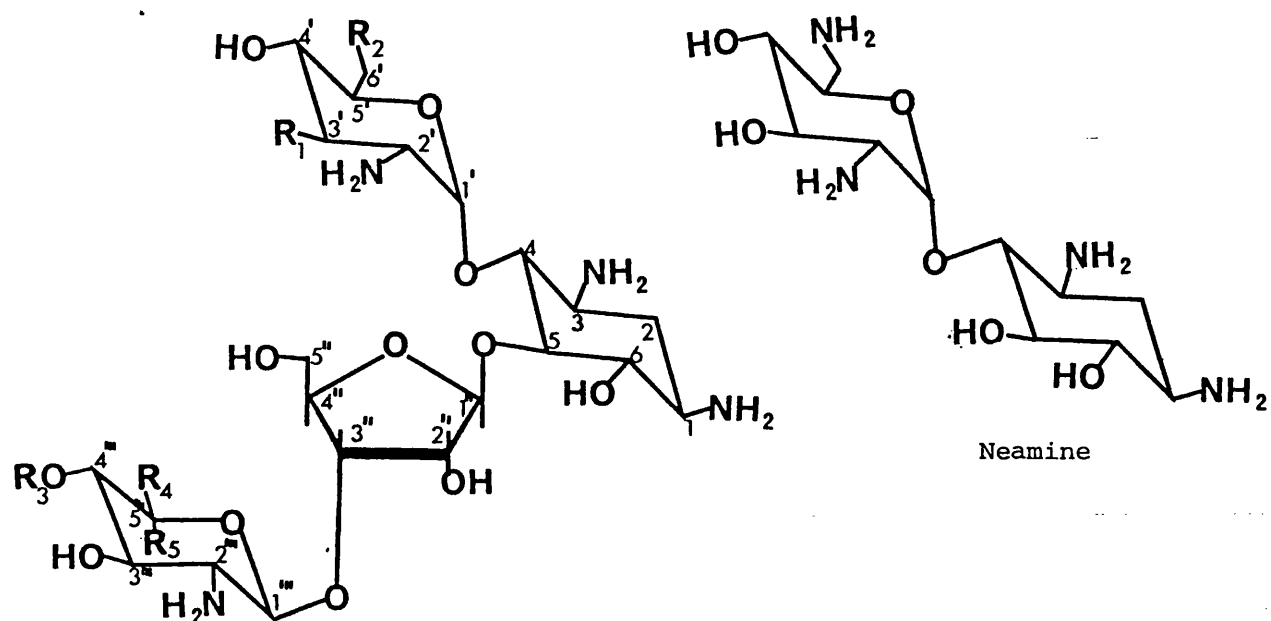
Figure 1.1. Structure of Streptomycin.



(1A) R = CHO Streptomycin

(1B) R = CH₂OH Dihydrostreptomycin

Figure 1.2. Structure of the Neomycins



	R ₁	R ₂	R ₃	R ₄	R ₅
Neomycin B	OH	NH ₂	H	H	CH ₂ NH ₂
Neomycin C	OH	NH ₂	H	CH ₂ NH ₂	H
Paromomycin I	OH	OH	H	H	CH ₂ NH ₂
Paromomycin II	OH	OH	H	CH ₂ NH ₂	H
Lividomycin A	H	OH	Mannose	H	CH ₂ NH ₂
Lividomycin B	H	OH	H	H	CH ₂ NH ₂

are required. Other methods have been devised to separate and assess component composition³²⁻⁴¹.

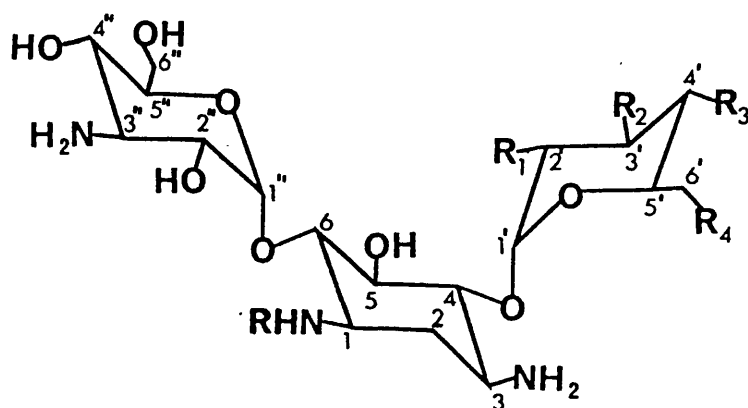
The paromomycins and lividomycins are very similar to the neomycins with the main difference being the degree of hydroxylation in the 'A' ring (Figure12).

Kanamycin also consists of three components (A, B and C) (Figure13)^{1,21}. Unlike the neomycins it is a 4,6 substituted aminoglycoside and only consists of three rings. Kanamycin A is the major component and is the least toxic¹. Kanamycin C is the least active whilst kanamycin B has twice the activity as well as twice the toxicity of kanamycin A. In the United Kingdom the amount of kanamycin B present in kanamycin sulphate BP is restricted to less than 3% w/v using the same ion-exclusion method as for framycetin sulphate BP but no limit is set for kanamycin C³⁰. In the United States there is also no limit on kanamycin C but a 5% w/v limit set on kanamycin B³¹, as measured by a selective bioassay. Other methods have been reported for kanamycin ratio determination⁴²⁻⁴⁴.

Tobramycin (Figure13), a member of the nebramycin complex, is 3'-deoxykanamycin B. Its purity is assessed by TLC and by its potency³⁰⁻¹.

Amikacin (Figure13)¹⁷, a commercially available semi-synthetic

Figure 1.3. Structure of the Kanamycins.



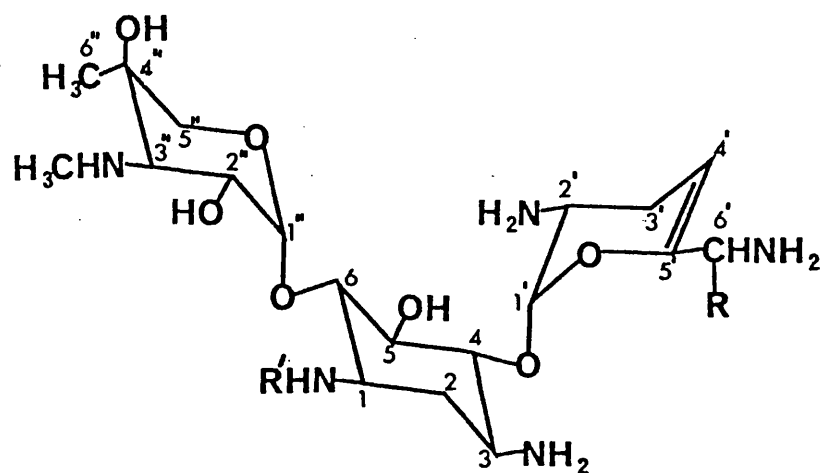
	R	R ₁	R ₂	R ₃	R ₄
Kanamycin A	H	OH	OH	OH	NH ₂
Kanamycin B	H	NH ₂	OH	OH	NH ₂
Kanamycin C	H	NH ₂	OH	OH	OH
Tobramycin	H	NH ₂	H	OH	NH ₂
Dibekacin	H	NH ₂	H	H	NH ₂
Amikacin	HABA	OH	OH	OH	NH ₂
Butakacin	HAB	OH	OH	OH	NH ₂

aminoglycoside is based on kanamycin A, having an L(-)- γ -amino- α -hydroxybutyric acid (L-AHBA) side chain attached to the C-1 carbon atom. This was synthesised because studies showed that the naturally occurring Butirosins were resistant to many inactivating enzymes, with the L-AHBA side chain present in that group of antibiotics being the probable protecting factor⁴⁵. Butakacin and Dibekacin (Figure 13) have also been synthesised to improve resistance to inactivating enzymes^{1,22,46,47}.

The sisomicins are a relatively recent family of aminoglycosides^{11,21,22}, with a novel structure (Figure 14) and show an antimicrobial spectrum similar to gentamicin^{1,48,2}. Sisomicin is the 4',5' dehydro derivative of gentamicin C_{1a}⁴⁹ and verdamicin exhibits a similar relationship to gentamicin C₂²¹. By selective N-ethylation of the C-1 amino group under controlled pH conditions netilmicin (Figure 14) was produced²⁰. It shows a similar potency to gentamicin but the N-ethyl group protects it against various important inactivating enzymes⁵⁰⁻¹. Other recently discovered aminoglycosides of importance include the seldomycins and the mutamicins^{46,52}, both of which show beneficial characteristics.

One of the largest families of aminoglycosides are the gentamicins^{1,9,22,52}. In the culture broth are produced many antibiotics. The gentamicin C complex is separated from other related compounds by use of a cation exchange resin to extract the antibiotics, followed by selective precipitation of the c-

Figure 1.4. Structure of the Sisomicins.

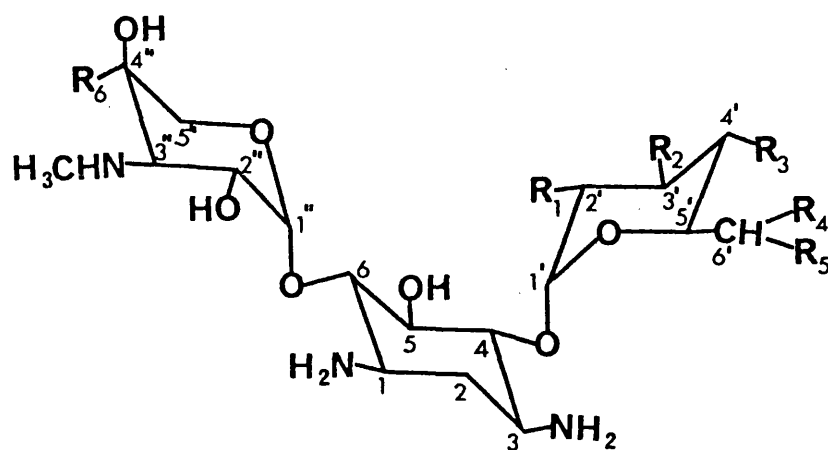


	R	R'
Sisomicin	H	H
Verdamycin	CH ₃	H
Netilmicin	H	CH ₂ CH ₃

complex using dodecyl sulphate^{21,53}. Co-produced antibiotics related to the c-complex are shown in Figure 15^{1,52}. They tend to be more polar and have quite extensive alterations in functional group substitution⁵⁴⁻⁶¹. Gentamicin A components have an aminopentose ring instead of the aminohexose (garosamine) subunit present in most other gentamicins^{58,59}. The 'A'-ring is the main site for the variation in structure of these polar components in respect to gentamicin C, with hydroxylation primarily at the 3' and 4' position increasing the polarity. Although they show lower toxicity than gentamicin C there is also a corresponding reduction in activity⁴⁶.

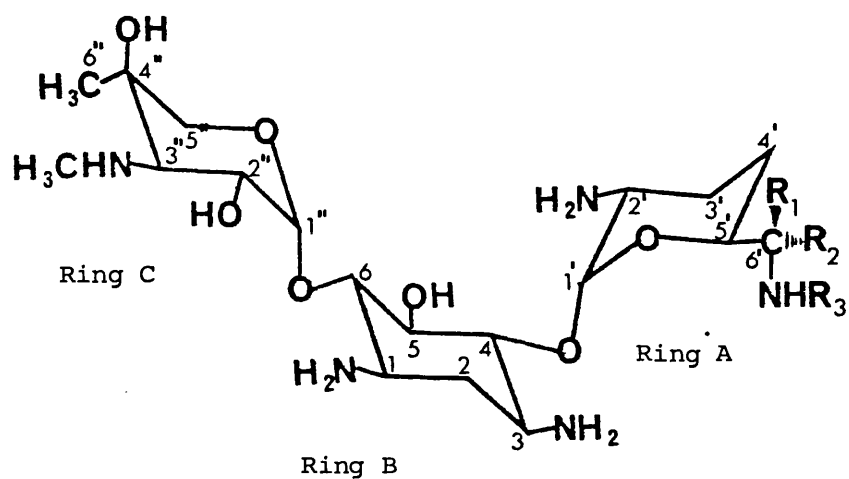
The gentamicin C complex is shown in Figure 16⁶²⁻⁶⁸. There are three main components C_1 , C_{1a} and C_2 ^{69,70} of which two additional isomers, C_{2a} and C_{2b} also exist^{16,71-73}. Ring C which is common for all five components is called the garosamine ring^{62,64} and ring A, the purpurosamine ring⁶⁵. The variations in the latter arise from the number and position of methyl groups present. Gentamicin C_{1a} has no methyl groups; C_2 , C_{2a} and C_{2b} each have one methyl group and gentamicin C_1 has two methyl groups in the A-ring^{63,65}. This is readily illustrated by proton NMR and mass spectrometry of these compounds⁶⁴⁻⁶⁸. The isomer⁷² gentamicin C_{2a} is thought to be an enantiomer of gentamicin C_2 whilst gentamicin C_{2b} is N-methylated instead of being C-methylated¹⁶. A further component having the L-manno configuration has also been reported⁷¹.

Figure 1.5. Structure of Gentamicin Polar Components.



	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
Gentamicin A	NH ₂	OH	OH	H	OH	H
Gentamicin B	OH	OH	OH	H	NH ₂	CH ₃
Gentamicin B ₁	OH	OH	OH	CH ₃	NH ₂	CH ₃
Gentamicin X	NH ₂	OH	OH	H	OH	CH ₃

Figure 1.6. Structure of Gentamicin C Complex



	R ₁	R ₂	R ₃
Gentamicin C _{1a}	H	H	H
Gentamicin C ₂	CH ₃	H	H
Gentamicin C _{2a}	H	CH ₃	H
Gentamicin C _{2b}	H	H	CH ₃
Gentamicin C ₁	CH ₃	H	CH ₃

Numerous minor components of the gentamicin series have been discovered with the majority belonging to the polar type compounds. These unfortunately show lower antimicrobial activity than the gentamicin C components and are susceptible to 3'-phosphorylating enzymes²². Other components of novel structure were discovered and interest has arisen as to the antimicrobial action and possible resistance to bacterial enzymes⁷⁴⁻⁷⁷.

The proportion of the gentamicin C components and levels of minor components is difficult to control in the commercial material. A wide range of geographical sources of the antibiotic has led to marketed samples showing significant compositional differences⁷⁶⁻⁷⁹. The desirability for a standard of the antibiotic is obvious and would restrict potential inequivalences^{47,78-9}. In the United States, limits are set on all three of the major components³¹:-

25 < gentamicin C₁ < 50%

15 < gentamicin C_{1a} < 40%

20 < gentamicin C₂ < 50%

The analysis of component ratio is achieved by paper chromatography to separate the components followed by a microbiological assay to quantitate levels. Thus the component composition is determined by potency estimation and has been criticised as being prone to inter-laboratory variation^{80,81}. The sample is also required to pass a potency and specific rotation test but it is apparent that mixtures with widely differing clinical

properties can be deemed suitable.

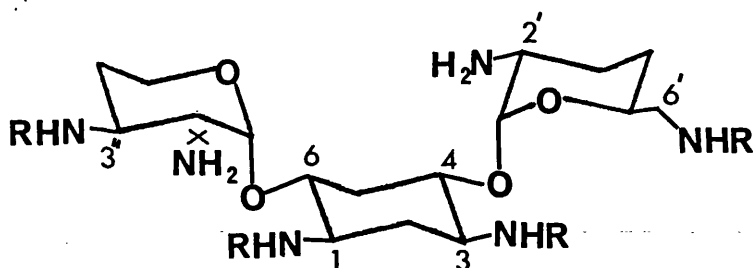
In the United Kingdom the component composition is not set as precisely as in the USA but is controlled within broad limits by a nuclear magnetic resonance (NMR) measurement of the sulphate salt in deuterium oxide^{30,81}. Quantification is by peak height measurement of the signals for methyl protons at different resonances. The mixture exhibits resonances due to two N-methyl groups, and two C-methyl groups and it is two ratios of these signals which define the limits. Only gentamicin C₁ concentration can be measured directly by this technique with the other two components expressed as ratios. This has been used successfully with commercial samples^{77,80,81} but is fraught with potential errors. Gentamicin C_{1a} is not measured directly and the presence of a component like gentamicin C_{2b} could lead to errors in gentamicin C₁ estimation. Minor components can interfere in the assay, and the total gentamicin C content is not measured.

A more specific and accurate analysis method for gentamicin is required especially to cope with the gentamicin C₂ isomers which have been reported in significant concentrations in gentamicin samples^{79,80,82-3}. Conventional thin-layer chromatography has been unable to identify these components⁸⁴⁻⁸⁸. High-performance liquid chromatography (HPLC) has been used in various studies in determinations of composition of material of varying geographical source^{78,79,82,89}. It has the advantages of speed, accuracy and

the ability to quantitate the gentamicin C_{2a} levels^{79,82}. Another useful method of component determination is field desorption mass spectrometry⁹⁰, but this requires specialised instrumentation which is not generally available.

Other methods have been proposed but are less adaptable than the HPLC method⁸³⁻⁸⁸.

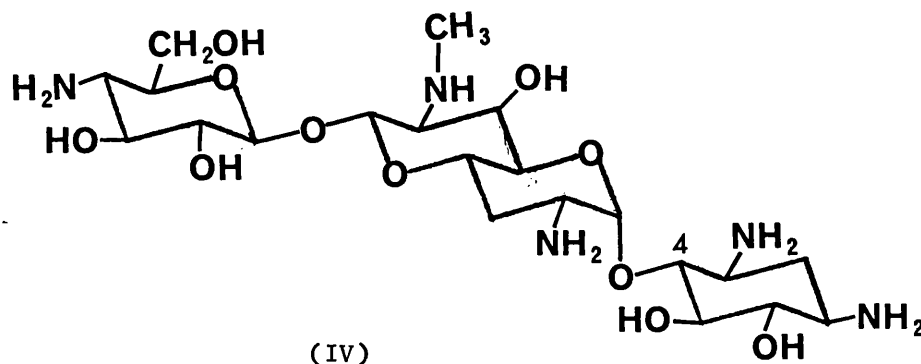
Structure-Activity Relationship



(III) Requirements for activity in 4,6 substituted glycosides

Due to the large number of aminoglycoside compounds discovered an extensive investigation into the influence of the structure of the antibiotic on its activity is possible (III)⁴⁶. It is known that the basic 2-deoxystreptamine subunit is inactive. Substitutions at the 5 and 6 position produces compounds with only weak activity whilst substitution at the 4 position also

gives weak activity, although apramycin (IV) has been found to be highly potent.



Thus highly active antibiotics in the main can only be obtained when 2-deoxystreptamine is disubstituted at the 4,5 or the 4,6 positions. Price⁴⁶ has discussed the requirements for activity in each of the subunits. The presence of amino functions has been found to be essential and for 4,5 substituted aminoglycosides the activity is increased by increasing the numbers of amino groups in the molecule. For 4,6 glycosides, at least in respect to the C-4 substituent, the activity is increased by an increase in amino groups present. Mono-N-alkylation with a small alkyl group like methyl has minimal effect on activity whilst N-acylation at any position except the amine at C-1 will reduce the activity. An amino group is required in the C-4 substituent but not in the C-5 substituent of deoxystreptamine. A primary or secondary amine is needed at C-3 and a basic function is required at C-1 in deoxystreptamine (III). In the C-6 substituent the location

of the amino group is essential in determining effectiveness in antimicrobial action. Information on hydroxyl contribution is less dependable and varies for different compounds. All hydroxyls can be modified in some manner yet retain activity, but various structural changes seen especially in the kanamycins can have a large influence on potency.

Therapeutic Aspects of Aminoglycosides

The aminoglycosides are active against gram positive and gram negative organisms as well as Mycobacteria^{1,2,50,91,92}. They are not against anaerobic bacteria or fungi. Some compounds (paramomycin and hygromycin B) possess antiprotozoal and anthelmintic activity⁴⁶ but these properties are not common to other aminoglycosides. Bacteria susceptible to aminoglycosides include *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus* sp., *Serratia* sp., *Klebsiella* sp., and *Staphylococcus aureus*². They do not possess significant activity against streptococci or meningococci. Gentamicin is the principal antibiotic for the treatment of many life-threatening infections such as septicaemia, neonatal infections, endocarditis, infected burns and peritonitis^{91,93-95}. Tobramycin is also used in a similar manner to gentamicin but amikacin is withheld for use against resistant organisms.

Mechanism of Action

The aminoglycosides are bactericidal but cell death is preceded by a marked inhibition of protein biosynthesis⁹⁶⁻⁹⁸. This activity is seen to occur at the 70s ribosomes but is incompletely understood. Streptomycin is known to bind to protein S12 of the 30s subunit involving at least four other ribosomal proteins and possibly 16s RNA⁹⁶. This binding is thought to induce conformational changes which disturb the normal functions of the acceptor and donor site⁹⁷. This is observed as misreading of the genetic code at the ribosome, inhibition of initiation of peptide chains on the complex, slowing of elongation of partly completed chains and increased unusual messenger activity⁹⁶. Gentamicin, neomycin and kanamycin show three separate concentration dependent effects on isolated ribosomes which suggest multiple binding sites for these drugs^{96,97}. These observations do not satisfactorily explain the lethal effect which may also involve damage to the cytoplasmic membrane⁹⁶. It is known that aminoglycoside uptake into bacterial cells occurs in three phases^{99,100}. There is an initial energy independent binding of the compounds to the exterior of the cell. This is followed by energy-dependent phase 1 in which aminoglycosides associate with membrane transporters by virtue of their positive charge and are driven across the membrane by the existing potential gradient. A faster rate of aminoglycoside uptake occurs after ribosomal binding. This is called energy dependent

phase II and the rationale behind it is not certain. It could either be due to synthesis of a polyamine permease⁹⁶ induced by the aminoglycoside itself or it could be due to an increased membrane potential associated with loss of potassium ions¹⁰⁰.

Phase II is essential for the lethal action of the aminoglycosides¹⁰⁰ to be realised, as is the ribosomal binding.

Resistance to Aminoglycosides

The build-up of resistance to the aminoglycosides is a problem especially associated with inappropriate drug use. It increased dramatically against streptomycin and kanamycin and was foreseen when gentamicin was introduced¹⁰¹. The frequency of resistant organisms to gentamicin in the United Kingdom has been comparatively low^{1,102} and careful antibiotic policy in hospitals has controlled outbreaks¹⁰³. There have been three possible mechanisms of resistance to aminoglycosides discovered^{47,100,104}; mutational change in ribosomal protein, reduced cell permeability and inactivating enzymes. The mutational change in the genes coding for ribosomal protein leads to the drug being unable to attach to ribosomes⁹⁶. This has arisen for streptomycin where replacement of a single amino acid in the S12 protein of the 30s ribosomal subunit can cause resistance⁹⁹. This type of resistance has not been shown to be clinically important^{100,105}. A second resistance mechanism, of gram negative bacteria, is a reduced

uptake of the aminoglycoside¹⁰⁰. Opinion has differed as to whether this is due to a change in the cytoplasmic membrane or due to enzyme production, as inactivated aminoglycosides do not accumulate in bacterial cells⁹⁶. A compositional change could involve replacement of magnesium ions by a protein H, preventing penetration of the membrane by aminoglycosides⁹⁹. This has been shown to be a clinically important type of resistance especially for *Pseudomonas* species and a major type of resistance⁵⁰ to amikacin. The third and most important form of resistance is due to aminoglycoside inactivating enzymes. The enzymes are classified by type of reaction they catalyze into aminoglycoside O-phosphotransferases (APH), O-nucleotidyltransferases, (ANT), and N-acetyltransferases (AAC)¹⁰⁰. Enzymes show specific action at defined sites and can be further classified for affinity for different substrates. Thus, AAC (3) I acetylates gentamicin readily at the C-3 amino group, but tobramycin only slightly, whilst AAC (3) II acetylates both gentamicin and tobramycin easily at the same site⁴⁷. Up to twenty one enzymes which modify aminoglycosides have been reported⁴⁷. Of these, about half act on gentamicin with different outcomes. The group of enzymes which act on gentamicin include AAC(3), AAC(2'), AAC(6'), APH(2'') and ANT(2''). Acetylation is a major route to inactivation due mainly to the deoxygenated nature of the gentamicin C complex. A similar number of enzymes inactivate tobramycin but act at different sites¹⁰⁶. The N-ethyl side chain of netilmicin is thought to offer protection against ANT(2''), APH (2'') and AAC(3)

enzymes^{22,51}. Similarly for amikacin the aminobutyric acid side chain protects the molecule from all enzymes except AAC(6')⁵⁰, in contrast to kanamycin A from which it is derived⁴⁷. Bacteria show variation in the type and number of inactivating enzymes they possess and this is reflected in the relative susceptibilities of these bacteria to the different aminoglycosides^{50,106}.

In gram positive and gram negative bacteria genes coding for these enzymes are carried on R-plasmids⁹⁶. It is these plasmids which have been responsible for the rapid growth in resistant bacteria, due to the ease of gene transfer by transduction and conjugation⁹⁷. The origin of R-factor resistant genes may have been in antibiotic producing organisms and even more information for aminoglycoside resistance could arise from that source¹⁰⁶.

Pharmacokinetics

The aminoglycosides are poorly absorbed by the gastrointestinal tract and thus are administered parenterally for systemic infections^{1,2,92,107}. Following intra-muscular injection of an aminoglycoside, peak serum levels are reached in thirty to ninety minutes depending on circulation and muscle mass¹⁰⁷⁻¹¹⁵. Administration by intravenous injection as a bolus produces immediate high peak plasma levels which soon recede^{2,107}. By

administering by iv. infusion over twenty to thirty minutes serum levels comparable to those obtained by intra-muscular injection are achieved². The half life of the aminoglycosides shows wide interpatient response but is about two hours^{107,108,116,117}.

A secondary half life has been calculated since it was observed that after treatment has finished, gentamicin continues to be excreted in the urine¹⁰⁷. The existence of a slower elimination pathway is supported by the lack of complete recovery of the dose in urine¹¹⁸, which should be possible since the aminoglycosides are unmetabolised. This secondary half life has been calculated to be about one hundred hours and may be due to release of drug from the renal cortex¹¹⁸. In renal failure the primary half life of two hours can extend to up to forty hours¹⁰⁹, since glomerular filtration is the major elimination mechanism¹¹⁸.

Haemodialysis removes the aminoglycoside¹¹⁹ and shortens the extended half-life considerably, in such compromised patients, and thus care has to be taken to ensure adequate drug levels^{109,110}.

Protein binding of aminoglycosides is not appreciable with values of 0 - 25% reported^{112,113,118}. The volume of distribution is about 25% indicating distribution of the drug throughout the extracellular water¹⁰⁷.

Dosages

The dosage interval of aminoglycosides is eight hours due principally to the two hour half life. The dosages for gentamicin, tobramycin and netilmicin are similar but amikacin requires much higher doses because of its lower potency¹. In children and infants a proportionally higher dose is required since aminoglycosides are well tolerated¹²⁰. This may be due to the higher percentage extracellular fluid volume which occurs in the very young^{121,122}. For gentamicin a dose of 2 - 5 mg/kg daily in three doses is recommended for an adult with normal kidney function¹²³. For children aged two weeks to twelve years a dose of 2 mg/kg is given every eight hours, whilst for infants less than two weeks old a dose of 3 mg/kg every twelve hours is recommended. In neonates the half life tends to be longer due to the lower glomerular filtration rate. In the presence of renal failure the dosage interval is usually lengthened in relation to the creatinine clearance^{92,109,116,119}. Various nomograms, relating body weight and creatinine clearance to an appropriate dose, have been proposed and used in clinical practice^{110,119,124-6}. They are useful for estimating a dose but plasma monitoring should be undertaken to ascertain the attained levels¹¹⁹.

For gentamicin and tobramycin a peak of 5 - 12 µg/ml and a trough of 1 - 2 µg/ml is desired for efficient treatment. For

amikacin the target peak concentration is 20 - 30 µg/ml and trough of 5 - 10 µg/ml⁹². Peak serum levels of less than 4 µg/ml of gentamicin or tobramycin and less than 14 µg/ml for amikacin are unlikely to be effective and could create resistant bacteria^{92,94}.

Therapy with other antibiotics can increase the effectiveness of the aminoglycosides. Indeed synergy with penicillins is well reported^{127,128}, such as the combination of gentamicin with carbenicillin in *Pseudomonas* infections¹²⁹. It is thought to arise from penicillin attack on the cell wall, increasing the penetration of the aminoglycoside. A secondary effect of the combined use of these antibiotics is that chemical inactivation can occur *in vivo* and *in vitro*¹³⁰⁻⁶. This is especially prevalent in intravenous infusion bottles¹³⁵, and when a high concentration of penicillin is present¹³¹. The reaction is thought to involve opening of the beta-lactam ring and subsequent reaction with the aminoglycoside^{132,135}.

Toxicity

The major disadvantage of the aminoglycosides is the low therapeutic index, with overdosage leading to severe side effects¹⁰⁷. It is known that the amino groups contribute significantly to the toxicity of aminoglycosides⁴⁶. Examples of this are found in the kanamycins. Kanamycin B has one more amino

group than kanamycin A and is twice as toxic. N-acetylation of any of the amino groups decreases the toxicity of the compound. The gentamicins A, X and B are thought to be less toxic than the gentamicin C components because they are hydroxylated at the 3' position. Price⁴⁶ related toxicity to antimicrobial activity and concluded that toxicity to micro-organisms could not be fully separated from the toxicity to mammals.

Toxicity is thought to be related to plasma levels although disagreement exists as to ascribing it to peak or trough levels, or frequency of dosing¹³⁷. Some studies have shown a significant correlation of trough levels greater than 4 µg/ml with a rising creatinine level^{138,139}. Another proposal is that susceptible people retain the drug in a three times higher concentration in the tissues than average¹¹⁸. For gentamicin it has been recommended for peak levels to be below 10 - 12 µg/ml and trough levels below 2 µg/ml.

The main adverse effects of aminoglycosides are nephrotoxicity, ototoxicity and neuromuscular blockade^{1,2,92}. The nephrotoxic and neuromuscular effects are usually reversible but the ototoxic effects are frequently irreversible^{138,140}.

Aminoglycosides are concentrated in the renal cortex with accumulation by reabsorption from the proximal tubule^{118,141,142}. This may arise by pinocytosis as this method is used by the brush

border cells for other compounds¹⁴². Alternatively they might be transported across the membrane by an active transport system¹¹⁸ or by binding to the charged anionic polyphosphoinositidies¹⁴³. These phospholipids are present in a relatively high concentration in the plasma membrane of renal cells and are believed to be involved in calcium transport¹⁴³⁻⁴. Complexation with these molecules could disrupt the membrane and increase the permeation of the gentamicin into the cell. It is suggested that 5 - 10% of the population show early and rapid tissue accumulation of the aminoglycosides, although the mechanism of this more rapid uptake has not been defined¹¹⁸.

Nephrotoxicity is gauged by a necrosis of proximal tubular cells associated with leakage of enzymes and proteins prior to an increase in serum creatinine¹⁴⁵⁻⁹. The onset of renal damage reduces the glomerular filtration rate¹⁴⁵ and thus leads to an accumulation of the aminoglycoside. The various aminoglycosides show differing toxic potentials with neomycin the most nephrotoxic⁴⁶. This may arise from the particular cellular toxicity of the drug rather than the extent of uptake¹⁴⁶. It may explain why netilmicin is considered to be less nephrotoxic than gentamicin even though rates of uptake are similar¹⁴⁶. The gentamicin C components are believed to have different toxicities although opinion is not agreed on which is the most nephrotoxic¹⁵⁰⁻².

Other factors which can precipitate acute renal failure are

dose and duration of therapy, state of hydration and underlying renal failure¹⁵³⁻⁴. Lack of standards of definition of nephrotoxicity restricts accurate estimation of occurrence.^{51,118} It is believed to occur in 2 - 10% of patients¹⁴⁸.

Ototoxicity, the other serious side effect of aminoglycoside treatment again is dependent on the particular drug but usually results in loss of inner ear function¹⁵⁵⁻⁶. The accumulation of aminoglycosides in the inner ear with slow elimination precipitates the toxicity⁹². Netilmicin is thought to be virtually non-ototoxic⁵¹ whereas gentamicin is significantly toxic⁴⁶. Gentamicin C₁ has been reported to be less ototoxic than the gentamicin C complex used as a whole¹⁵⁵. A theory of interaction with the phospholipids of the cellular membrane has been proposed for the toxic effect^{141,157}. Vestibular function can also become disturbed and is associated with ototoxicity⁹².

Neuromuscular blockade has been shown to be reversible with calcium ions and to be similar to that produced by conventional blocking agents⁹⁸. This can induce weakness of skeletal muscle and respiratory depression.

To minimise toxic effects, careful use in clinical practice, especially in susceptible patients, is required. Monitoring of plasma levels is required in these patients to prevent onset of toxicity^{119,137}.

Plasma Analysis of Gentamicin

The assessment of plasma levels of gentamicin to optimise treatment¹⁵⁸ has traditionally fallen to the microbiological assay; either by diffusion or by dilution^{137,159-164}. The dilution method was shown to be highly inaccurate in a quality control survey and is little used now^{163,165}. The plate diffusion method can use either well or disc for distribution of antibiotic to the medium¹³⁷.

The procedure is subject to large variation due to type of medium used¹⁶⁶⁻⁸, concentration of cations, species of organism tested against, pH¹⁶⁷ and composition of sample¹⁶⁷⁻⁹ and standard solutions and the length of time of incubation¹³⁷. The time required for an assay is a major disadvantage with twelve to eighteen hours required for zone diameters of inhibition to be measured¹³⁷. Clinical signs of inappropriate treatment may therefore be available before the plasma concentration is known^{159,161}. Faster bioassays were developed to achieve a measurable zone in three to four hours using a heavy inoculum of a fast growing species of bacterium^{159,170-2}. Principal advantages of the bioassay are that it is a cheap and easy technique requiring skills readily available in hospitals¹⁷³. It is also very flexible in adaption to size of workload. A disadvantage is the poor accuracy which was highlighted in quality control surveys carried out in 1973 and 1974.

Only 21% of those tested achieved a $\pm 25\%$ accuracy¹⁶⁵. The presence of other antibiotics in the sample can interfere with results especially when the laboratory is not informed of them^{158,174}. Some assay media have also shown a tendency to underestimate gentamicin levels in uraemic sera¹⁶⁹. In comparison to other assay methods the bioassay was held to be clinically acceptable^{173,175-80} except in one case¹⁸¹. They generally exhibited poorer precision and correlation with other methods was variable^{177,180,182}.

A quick and simple technique utilises gentamicin-sensitised latex particles which react with anti-gentamicin antiserum to give an agglutination reaction¹⁸³⁻⁵. It is ideal for low workloads although performance at plasma concentrations of less than 2 $\mu\text{g/ml}$ is poor¹⁸⁴⁻⁵.

The radioenzymatic method, a much used technique, involves the acetylation or adenylation of gentamicin by bacterial enzymes¹⁸⁶⁻¹⁹⁰. The coenzyme in either case (ATP or acetyl CoA) is tagged with a radioactive label¹⁵⁹, which after reaction produces a labelled gentamicin derivative. This is adsorbed onto phosphocellulose discs and counted on a liquid scintillation counter¹³⁷. The assay method is quick, requiring two hours to perform and is relatively easy to use. However stability problems of enzyme, breakdown of ATP by inherent ATPase in serum and variations in binding capacity of the phosphocellulose paper

have been noted¹³⁷. There were reports that plasma constituents and tetracyclines¹⁹¹ have decreased gentamicin readings.

Availability of a scintillation counter is essential and expertise in the handling of radioactive isotopes is a requisite¹³⁷. In clinical studies^{173,175,179,182} the radioenzymatic method was satisfactory except on one occasion where the kit was judged to be unacceptable¹⁹².

Immunoassays developed for the aminoglycosides use radioactivity, absorbance and fluorescence as an indicator of extent of binding.

In radioimmunoassays¹⁹³⁻⁴ gentamicin from the sample competes with standard labelled gentamicin for antibody binding sites decreasing the measured bound activity¹⁹⁵⁻⁷. The assay is specific, sensitive and precise and results are obtained in two hours¹⁶¹. The cost of reagents is high¹⁶² and is comparable to those used for fluoroimmunoassays^{176,182}. In three clinical studies^{176,182,192} the readings were found to be consistently high which has been ascribed to significantly inaccurate standards^{192,198}. The precision of the assay is, like other immunoassays, more than adequate for clinical needs^{176-8,180-2,193,199}. Similar equipment to that used in the radioenzymatic method is required.

Enzyme immunoassays²⁰⁰⁻⁶ generally involve competitive binding to antibody, of gentamicin in the sample, with

gentamicin-linked enzyme in the standard. The extent of binding is revealed as a change in the activity of the enzyme. One of these kits, EMIT (Syva Corporation)²⁰⁶ utilises a gentamicin-glucose-6-phosphate-dehydrogenase conjugate (G6PDH) which can convert nicotinamide adenine dinucleotide (NAD) to NADH, a change which can be monitored by UV spectrophotometry.. The assay requires a spectrophotometer with a thermostatted flow cell and a timer-printer, as readings are made after 15 and 45 seconds²⁰⁶. Cross reactivity is only a problem with other aminoglycosides and therefore unlikely to be significant in clinical practice²⁰⁵. The assay is easy and very fast with only thirty minutes required for a calibration¹⁷⁶. A loss of precision has been noticed at the higher end of the concentration range but can be overcome by diluting the plasma²⁰⁷. The EMIT method has been assessed in various papers^{176-9, 181-2, 192, 199} and no detrimental properties were reported. Precision and specificity were high although in one paper, recovery was low¹⁷⁹. The cost of reagents and equipment was considered to limit the technique to large numbers of samples^{176,182}.

Fluoroimmunoassays for aminoglycosides²⁰⁸⁻¹³ mainly depend on quenching^{209,213} or polarisation^{208,212} of fluorescence when gentamicin labelled with fluorescein is bound by antibody. The two methods, as well as EMIT, are termed homogenous because no separation step is required. An increase in polarisation of fluorescence results when fluorescent ligand binds to the antibody

because of an increase in rotational relaxation time^{208,212}.

Thus gentamicin in the sample will cause an increase in fluorescence in one assay and a decrease in polarisation in the other.

Standard curves used in these assays were not straight lines raising the question of accuracy^{208,209}. Another fluoroimmunoassay uses an enzyme which reacts with a gentamicin-conjugate to produce a fluorescent species, which is quenched by binding to antibody²¹⁰⁻¹¹.

The fluoroimmunoassays, similar in cost to the other immunoassays, were generally less precise than EMIT in most studies^{176-78,180-2}. One paper found apparent gentamicin readings due to penicillin in one kit¹⁸². Direct correlation of the results with those obtained by enzyme immunoassay was good but it was not as fast or as easy to use¹⁸². For both fluorescence and enzyme immunoassays it is recommended that calibrations be made each day thus adding to the overall cost¹⁷⁶.

A potential disadvantage of the immunoassays is the possibility of non-specific binding of substrate labelled drug to plasma constituents, such as protein²¹⁴. Experience of the clinical use of these assays suggests this is of little significance²¹².

Background fluorescence of plasma could interfere with fluorescence measurements²⁰⁹ but again has not been shown to be important¹⁷⁷⁻¹⁸⁰.

Another method used for aminoglycoside analysis in plasma

is chromatography^{162,215-8}. Gas-liquid chromatography (GLC) was used, after derivatisation of gentamicin, but the C_{1a} and C₂ components were not separated²¹⁹.

High-performance liquid chromatography (HPLC) has been used experimentally many times for this analysis with many methods developed²²⁰⁻³⁵. The popularity of the technique in relation to GLC originates from the different conditions used to effect separation. For HPLC, chromatography of non-volatile compounds is possible, separations can be varied enormously, sample preparation can be easy and specificity of detectors can isolate drugs from the background matrix²¹⁵. The HPLC technique was compared in three studies with various other assays¹⁷⁸⁻⁸⁰. In one study HPLC was found to have the worst precision when compared with the three immunoassays but was still thought to be satisfactory¹⁷⁸. In another study HPLC showed the best precision¹⁷⁹. Both these papers noted that the labour intensive nature of HPLC was a major drawback. The final paper found no specific advantage for HPLC and indeed precision was poor although the fluoroimmunoassay was equally imprecise¹⁸⁰. Thus in these general studies the HPLC method was not found to possess any substantial advantages. However they ignore the unique ability of HPLC to quantitate individual component levels for gentamicin. This is of potential use if the components are discovered to differ in pharmacokinetic or toxic properties. The low comparative cost of the HPLC reagents is counteracted by increased

staff cost. The latter could be reduced if automation could be introduced to the technique.

Thus, there have been many methods developed for the analysis of aminoglycosides in plasma reflecting the requirement for fast and accurate measurements. None of these techniques shows overwhelming success and each is satisfactory in some respects.

In the present study it was intended to develop a method for the accurate analysis of gentamicin levels in plasma using HPLC. It was proposed to adapt the method for the assay of gentamicin components in commercial gentamicin sulphate samples and pharmaceutical preparations. In pursuing this it was found necessary to investigate an existing inconsistency in the current literature concerning the HPLC behaviour of gentamicin components.

In addition it was proposed to isolate minor components of the gentamicin complex from commercial gentamicin samples, to characterise these using spectroscopic and chemical techniques and to investigate possible antimicrobial activity.

CHAPTER 2

ISOLATION AND IDENTIFICATION OF MINOR COMPONENTS OF THE

GENTAMICIN SERIES

2.1 Introduction

The determination of aminoglycoside structure was, in the past, accomplished by classical chemical techniques of oligosaccharide analysis^{24, 25, 244}. Recent improvements in instrumental analysis have enhanced the structural resolution of mixtures of aminoglycosides^{49, 62-66, 245}.

Carbon-13 nuclear magnetic resonance spectroscopy has proved to be particularly useful in this respect²⁴⁶⁻⁷. The wide range of chemical shifts and use of off-resonance decoupling enable individual carbon atoms to be identified. The similarity of the chemical shifts of the equivalent rings in different aminoglycosides has been considered advantageous, and the use of protonation shifts for the determination of the stereochemistry of the sugar groups in relation to the deoxystreptamine ring has also proved to be useful²⁴⁷. However, the ability of aminoglycosides to absorb carbon dioxide from the atmosphere can produce erroneous results²⁴⁶.

Proton nuclear magnetic resonance spectroscopy (NMR) is less applicable to the aminoglycosides than carbon-13 NMR because of the large number of protons resonating in a smaller shift range. Extensive overlap of proton resonance peaks occurs, masking useful structural information. Degradation to the single ring moieties

followed by proton NMR has proved very useful in providing conformational information⁶⁴⁻⁶⁶.

Mass spectrometry is an ideal method for size and structure analysis especially with small amounts of sample²⁴⁸. Electron impact (E.I.) mass spectrometry²⁴⁹ has been widely used for the aminoglycosides. The various fragmentation pathways of the gentamicins were characterised and these have also been applied to other aminoglycosides^{20, 67, 68}. Electron-impact relies on sample volatisation before ionization by a beam of electrons. This mechanism produces low ion yields for polar and nonvolatile compounds such as the aminoglycosides. Adaptations of the basic technique have been attempted to increase ion yield²⁵⁰. Apart from these methods excessive heating is normally required for the aminoglycosides and although structurally useful fragments are produced the molecular ion yield is very low⁶⁸.

Chemical Ionization (C.I.)²⁴⁹ is a "softer" method of ionization than E.I., relying on ion-molecule reactions, with the ions generated from a reactant gas. The particular gas used can produce an individual fragmentation pattern. The initial energy content of molecular ions formed by CI is generally much less than for EI and this results in less fragmentation and a higher yield of molecular ion. For the aminoglycosides fragmentation is usually only into the major subunits and a clear indication of structure is achieved^{77,90}.

Field desorption (F.D.) mass spectrometry²⁵¹ also uses a "soft" ionization, where volatilisation of the sample occurs after ionization and depends on the electrical field strength and the emitter anode temperature^{249, 252, 253}. Protonated and cationized pseudo-molecular ions can form and are usually more stable than the molecular ion^{250, 254}. Spectra produced by F.D. show predominantly such pseudo-molecular ions with very little fragmentation, and the method is thus suitable for molecular weight determinations of unknown components⁷⁷. Polarity and volatility of the sample is not as critical as E.I. but molecular ion yield is low and transiently produced²⁵⁵⁻⁷.

More recently other "soft" ionization methods have been developed. These include plasma desorption^{254, 258-9}, electro-hydramic ionization²⁶⁰⁻², laser desorption²⁶³, secondary ion²⁶⁴⁻⁶ and fast atom bombardment mass spectrometry^{255-6, 267-72}. A localised hot spot which enhances desorption of ions has been proposed to occur with these methods and similar spectra can be observed between them even though particles of vastly differing energies are used to achieve ionization^{250, 254}. Both positive and negative ion spectra can be recorded by these methods which can extend their utility.

Fast atom bombardment (FAB) is a development of secondary ion mass spectrometry and indeed some suggest there is little difference between them²⁶⁵⁻⁶. Instead of a beam of ions, a beam of atoms, of usually xenon or argon, is used to produce secondary ions from the sample. The sample is suspended in a liquid, usually

glycerol, and is bombarded by atoms at about 20° to the horizontal to give maximal ion yield^{254, 271}. The glycerol phase probably presents a constantly new surface for bombardment, by diffusion and thermal currents enabling stable ion production over a relatively long period of time²⁷⁰. Some compounds are enriched at the surface, whilst others can be completely masked by the glycerol²⁵⁴. Xenon used as a source gas can increase ion yield probably by virtue of its larger molecular size²⁷¹. FAB has been used for numerous non-volatile compounds^{255-6, 272} such as peptides, and even gentamicin sulphate was measured by this method²⁶⁹. The combination of high molecular ion yield with some fragmentation promises the versatility required for analysing minor components of the gentamicin series.

The minor components were isolated from commercial gentamicin sulphate of various origins by column chromatography. Characterisation of these components was primarily achieved by mass spectrometry with NMR used for larger sample sizes.

2.2 Materials and Methods

2.2.1 Materials

Gentamicin sulphate samples from a variety of geographical sources were donated by Nicholas Laboratories Ltd. Netilmicin sulphate was supplied by Kirby-Warrick Pharmaceuticals Ltd. Silica gels for column chromatography of different particle size were used. Woelm silica gel, less than $63\text{ }\mu\text{m}$ and $100 - 200\text{ }\mu\text{m}$

particle size were obtained from Koch Light Laboratories Ltd. Silica gel, 15 - 40 μm and silica gel H for thin-layer chromatography were from Merck, supplied by BDH Chemicals Ltd. Glass columns with fixed internal glass sinters and dimensions of 45 x 5½, 40 x 2, 30 x 1½ and 30 x 1 cm were used. Ninhydrin and dimethylamine borane were obtained from Aldrich Chemical Company Ltd. Amberlite IRA 400 (Cl) resin was from BDH Chemicals Ltd. General purpose grade solvents were redistilled before use.

2.2.2 Methods

Thin-Layer Chromatography (T.L.C.)

Silica gel 60 plates were made as required. They were spread on 10 x 20 degreased, glass plates with a layer thickness of 0.25 mm. The developing solvent was the lower layer of a chloroform/methanol/ammonia (2:1:1 (18 M)) mixture. The tank was allowed to equilibrate for at least three hours before use. New mobile phase had to be prepared every four days due to evaporation of ammonia causing decreased solvent strength. A sample volume of 5 - 10 μl was applied for each spot using a micro syringe. Development of the plates was continued until the solvent had travelled at least 10 cm. Plates were dried at 110°C for twenty minutes to remove ammonia. Detection was by use of ninhydrin spray, consisting of ninhydrin (1.2 g) dissolved in pyridine (50 ml) and acetone (50 ml). After spraying the plates were heated at 110°C for five minutes to produce purple-red spots on a white background.

Paper Chromatography

A descending technique using Whatman No. 1 paper and a solvent system of the lower phase of a mixture of chloroform/methanol/ammonia (2:1:1 (9M)) was used. Both upper and lower phases were allowed to saturate the tank for twelve hours before development. The solvent took five hours to reach the end of the paper during development, after which, the paper was dried for twenty minutes at 110°C. Spots were detected with the same ninhydrin spray used for TLC.

Preparation of Gentamicin Base

Gentamicin base was generated from the sulphate salt by use of an anion exchange resin, Amberlite IRA 400. Resin (Cl) (50 g) was converted first to the OH⁻ form by passing 1M sodium hydroxide through the resin. Distilled water was used to wash column free of alkali. Gentamicin sulphate (4 g) was dissolved in a minimum amount of water, passed onto the column and eluted with water. Alkaline fractions were collected and lyophilised to yield gentamicin free base. This was stored (over phosphorous pentoxide) in a desiccator together with potassium hydroxide pellets to absorb carbon dioxide.

Column Chromatography of Minor Components

Various columns, types and weights of silica gel were used. In general, silica gel (40 g) was slurried in the mobile

phase and packed into a column of 40 x 2 cm dimensions, with internal volume of 125 ml. The column was allowed to stand for twenty four hours before use to allow trapped air to escape. The initial eluent was the lower phase of a chloroform/propan-2-ol/2.25 M ammonia (2:1:1) mixture. Gentamicin base (6 g) was dissolved in about 10 ml of the eluent and applied directly to the top of the column. Elution proceeded at about 0.3 ml min^{-1} for about three column volumes when the eluent was changed to the lower phase of a (2: 1:1(4.5 M)) mixture of the same solvents. This was continued until gentamicin C_1 was initially detected (by TLC monitoring of fractions). The solvent polarity was then increased to (2:1:1 (9M)) and continued until gentamicin C_2 began to elute. Methanol was then used to elute the remaining components.

When the major components were to be collected separately, methanol was not used. Instead, the elution was continued with a (2:1:1 (18M)) mixture followed by a (1:1:1 (18M)) mixture after gentamicin C_2 had eluted.

2

Fractions were collected in 5 ml aliquots on a LKB 7000 fraction collector, which could be adjusted to step at specific time intervals as required. Fractions were monitored by Thin-Layer Chromatography, comparing with a standard 1% w/v gentamicin sulphate solution, and those of similar composition were combined and the solvent removed under reduced pressure. Residues were redissolved in a small amount of distilled water and then lyophilised. Components collected were stored in

desiccators over silica gel or phosphorus pentoxide and potassium hydroxide.

Semi-Preparative High Performance Liquid Chromatography (H.P.L.C.)

A single run on a Jobin Yvon Mini-Prep system was completed. The mobile phase used was the same as for column chromatography except that elution was started with the lower phase of a (2:1:1 (9M)) mixture. Silica gel used for the stationary phase was Lichroprep Si 60, 15 - 25 μm . To construct a column of size 37 x 2 cm, required 50 g of silica. Flow rate was 1.8 ml min^{-1} at a solvent pressure of 8 bar. Fractions of 3 ml were collected on a fraction collector. Monitoring of the eluate was performed by TLC.

Hydrolysis:

Hydrolysis⁶⁵ of major components was accomplished by dissolving the sample (100 mg) in 6 N hydrochloric acid (20 ml) and refluxing for six hours. The product, 2-deoxystreptamine dihydrochloride was isolated by precipitation when the hydrolysate was added, dropwise, to dry ether (250 ml). The precipitate, isolated by filtration, was purified and converted to its base form by passing through an anion exchange column.

N-Acetylation: Micro-scale N-acetylation was carried out as follows. Sample (0.5 - 5 mg) was dissolved in methanol (100 μ l) in a reaction vial. This was placed in ice and acetic anhydride (50 μ l) was added dropwise to the cooled methanolic solution. After stirring with the needle of the syringe the reaction was left for three hours at room temperature, Progress of reaction was followed by the TLC system. On disappearance of ninhydrin positive spots the reaction was judged to be complete. The reaction was terminated by adding 18 M ammonia (60 μ l). This solution was passed through a small column (5 g) of Amberlite IRA (OH) 400 resin, and the eluate lyophilised.

Mercaptolysis:

The procedure of Cooper et al.⁶⁵ of mercaptolysis of the N-acetylated components was adopted. In this the compound (10 mg) was dissolved in ethanethiol (100 μ l) and concentrated hydrochloric acid (100 μ l). The reaction was left for 24 hours at room temperature after which, the thiol was evaporated in vacuum at 40°C. The remaining solution was diluted with water (10 ml), neutralised by addition of lead carbonate, filtered and lyophilised. The residue obtained was dissolved in methanol (100 μ l) and acetic anhydride (50 μ l) was added dropwise. After six hours at room temperature, the solvent was removed by vacuum and the residue dissolved in chloroform. The solution was filtered to remove N,N-diacetyl-deoxystreptamine and chromatographed on a silica gel column (20 g) using toluene/methanol (4:1) as the mobile phase. The main component

as determined by TLC was collected and the solvent removed by evaporation. Mass spectrometry was used to identify the fraction as a N-acetylpuerpurosamine thioacetal.

Reduction:

The reduction of one of the components suspected of containing an imino group was also carried out. The sample (20 mg) was dissolved, in a reaction vial, in glacial acetic acid (40 μ l). Dimethylamine borane (10 mg) was dissolved in glacial acetic acid (60 μ l) and 20 μ l of this solution was added to the vial, and the mixture stirred with the needle of a microsyringe. The reaction was left for two hours at room temperature, then 18 M ammonia (60 μ l) was added. The solution was passed through a small anion exchange column and the eluate lyophilised.

Mass Spectrometry:

Field desorption mass spectra were determined by Mr. M. Rossiter at the Department of Chemistry, University College, Cardiff using a Varian CH-5D mass spectrometer. Chemical Ionization, Electron Impact and Fast Atom Bombardment mass spectra were determined by Mr. C. Cryer at the Department of Chemistry, University of Bath, using a V.G. 7070 E mass spectrometer.

Nuclear Magnetic Resonance (N.M.R.)

The 100 MHz proton NMR spectra were determined by Mr. D. Wood on a JEOL P.S. 100 NMR spectrometer and carbon-13 NMR spectra were determined by Mr. H. Hartnell on a JEOL FX 90Q Fourier Transform NMR spectrometer at the School of Pharmacy and Pharmacology, University of Bath.

2.3 Results and Discussion

2.3.1 HPLC Semi-Preparative Isolation of Minor Components

The separation obtained from one run of a Jobin-Yvon instrument using 1.5 g of gentamicin base was compared to the separation of 0.5 g of gentamicin base by a gravity fed column in Table 2.1.

With both columns some overlap of major components occurs but the resolution of the Jobin-Yvon column was superior to that of the ordinary column. The semi-preparative instrument also used less mobile phase and was more rapid. This advantage in speed was negated to an extent by the TLC method of column monitoring which increased the run time of the column considerably and was by nature a retrospective check. The UV monitor of the eluent was inappropriate with gentamicin because of the poor absorption characteristics. A refractometer would have been more suitable but was unavailable.

The semi-preparative instrument coupled with an equally matched detection system would prove to be far superior

Table 2.1. Comparison of Semi-Preparative HPLC with Classical
Column Chromatography

Component	Jobin-Yvon Elution volume (ml)	Gravity Column Elution Volume (ml)
Minor	105-192	225-390
Gentamicin C ₁	204-330	414-660
Gentamicin C ₂	315-453	576-750
Gentamicin C _{1a}	480-570	720-900
<u>Mobile phase</u>		
2:1:1 (9M)	0-390	0-730
2:1:1 (18M)	391-690	731-1380

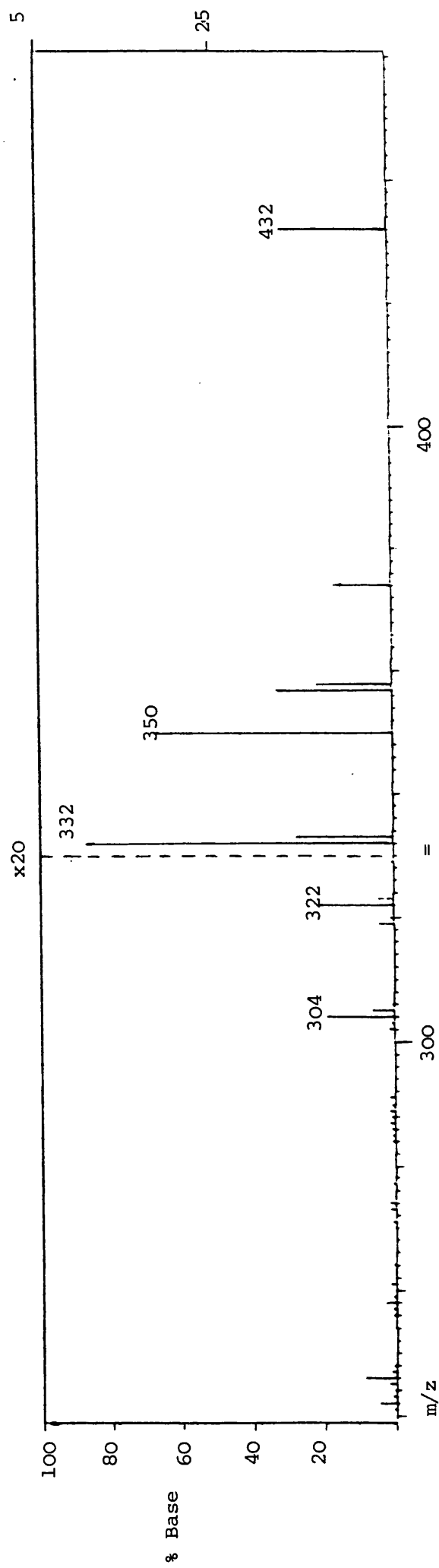
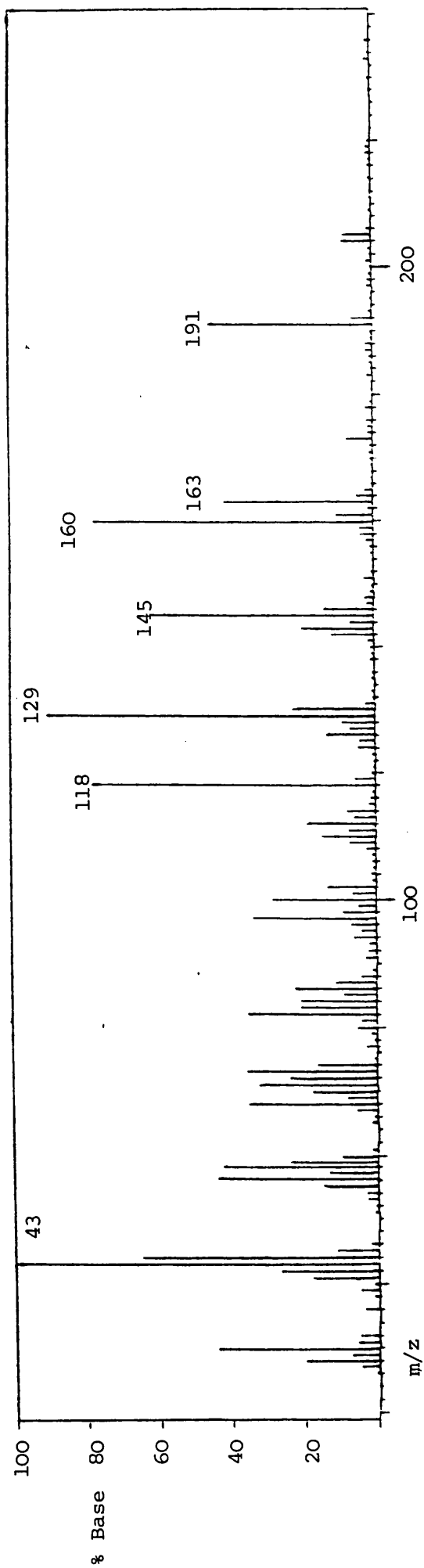
to the ordinary classical method, yet the TLC used here decreased the advantages.

2.3.2 Spectral Characteristics of the Gentamicin C Components

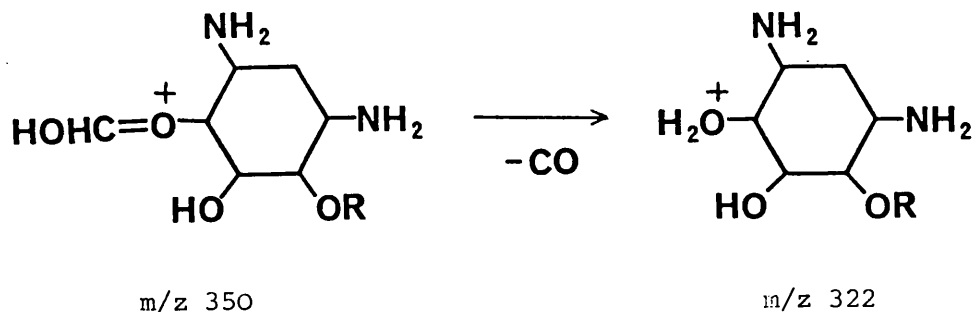
Mass Spectrometry:

Electron impact spectra of all three major components are similar to that illustrated in figure 2.1 for gentamicin C_{1a}. The fragment ions were identified by Daniels et al.^{67,68} and are reproduced here. There is no molecular ion in figure 2.1 but the peak at m/z 432 is probably due to loss of ammonia from that ion (m/z 449). The peaks at m/z 350 and 322 are highly characteristic of the gentamicins and represent two ring

Figure 2.1. EI Mass spectrum of Gentamicin C_{1a}



fragments:

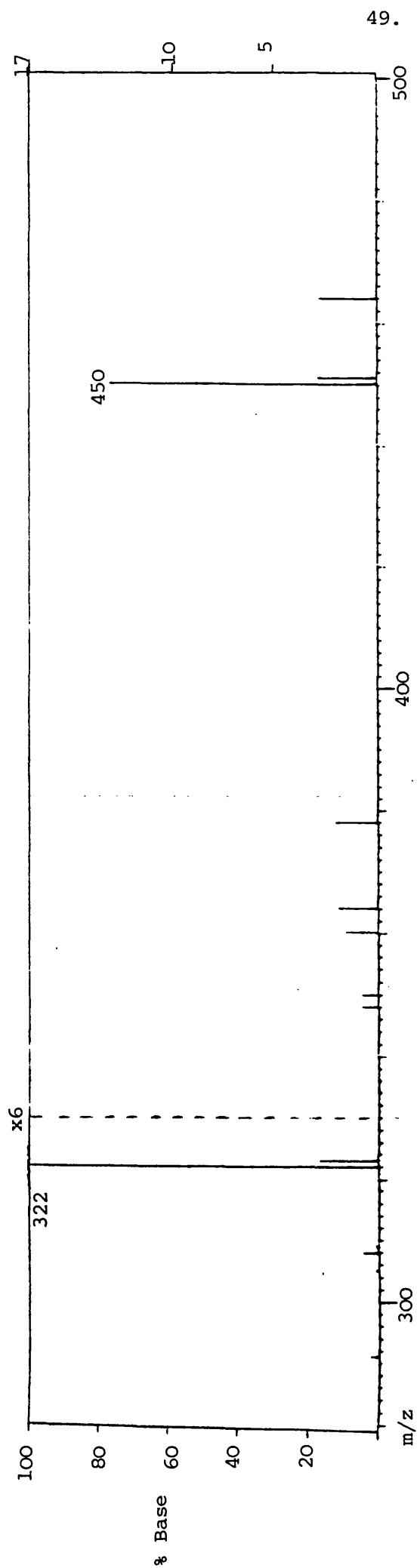
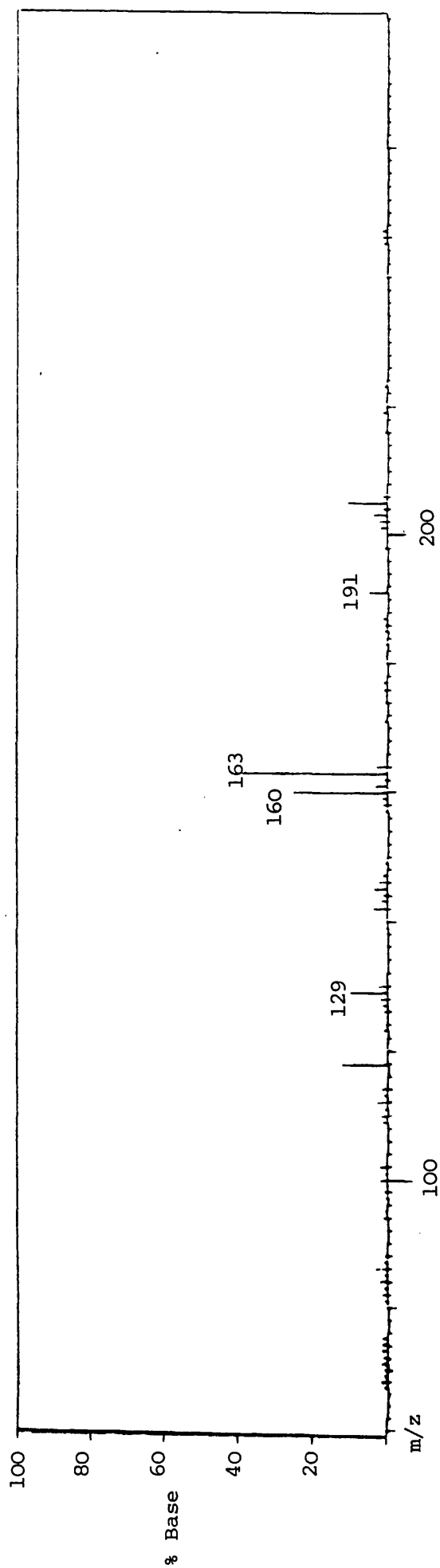


R = Garosamine

The fragment, m/z 322, can further lose water to give another fragment at m/z 304. These three ring fragments are indicative of a garosamine-deoxystreptamine combination. The presence of 2-deoxystreptamine can be further verified by the peaks at m/z 191, 163 and 145⁶⁸. Garosamine is indicated by a fragment of m/z 160 which can lose water to give m/z 142. The A-ring of the gentamicins which is preferentially cleaved off is shown by a peak at m/z 129 in figure 2.1. Gentamicins C_1 and C_2 show corresponding peaks at m/z 157 and 143 respectively, for the purpurosamine A and B fragments. More extensive fragmentation of these subunits produces prominent peaks at m/z 130, 126 and 118. Electron impact spectra can often provide comprehensive structural data but often at the loss of the molecular ion. This is disadvantageous when investigating component of unknown molecular weight.

Chemical ionization spectra show the peaks due to the fragments of the three subunits but little other fragmentation.

Figure 2.2. C.I. Mass Spectrum of Gentamicin C_{1a}



The yield of the pseudo-molecular ion $[M+H]^+$ is large as shown in Figure 2.2 which is the CI spectrum of gentamicin C_{1a} .

A field desorption spectrum of gentamicin C_1 is shown in Figure 2.3 and reveals just the pseudo-molecular ion with traces of impurity present.

The fast atom bombardment spectrum of gentamicin C_{1a} with some gentamicin C_2 present is illustrated in Figure 2.4. This is a positive ion spectrum because negative ion yield was low with the instrument used. The $[M+1]^+$ peak is evident for both of these components in the spectrum. Also present is the $(M+sodium)^+$ peak at 472 for gentamicin C_{1a} . This is a common occurrence with FAB, and also sometimes with FD, glycerol can also form complexes with ions which can complicate the spectrum immensely. There is fairly extensive fragmentation in this spectrum comparable to that produced by CI. As a whole the FAB technique is potentially the most versatile of those discussed for polar compounds.

To further aid identification, N-acetylation can be used to produce FAB spectra similar to that shown for the gentamicin components in Figure 2.5. The penta and tetra N-acetyl derivatives of each component are present, as are the N-acetyl derivatives of the purpurosamines at m/z 241, 227 and 213. Garosamine and deoxystreptamine fragments are at m/z 202 and 247 with a combined fragment at m/z 448. N-acetylation is thus a very useful technique for determining individual ring constituents.

Figure 2.3. F.D. Mass Spectrum of Gentamicin C₁

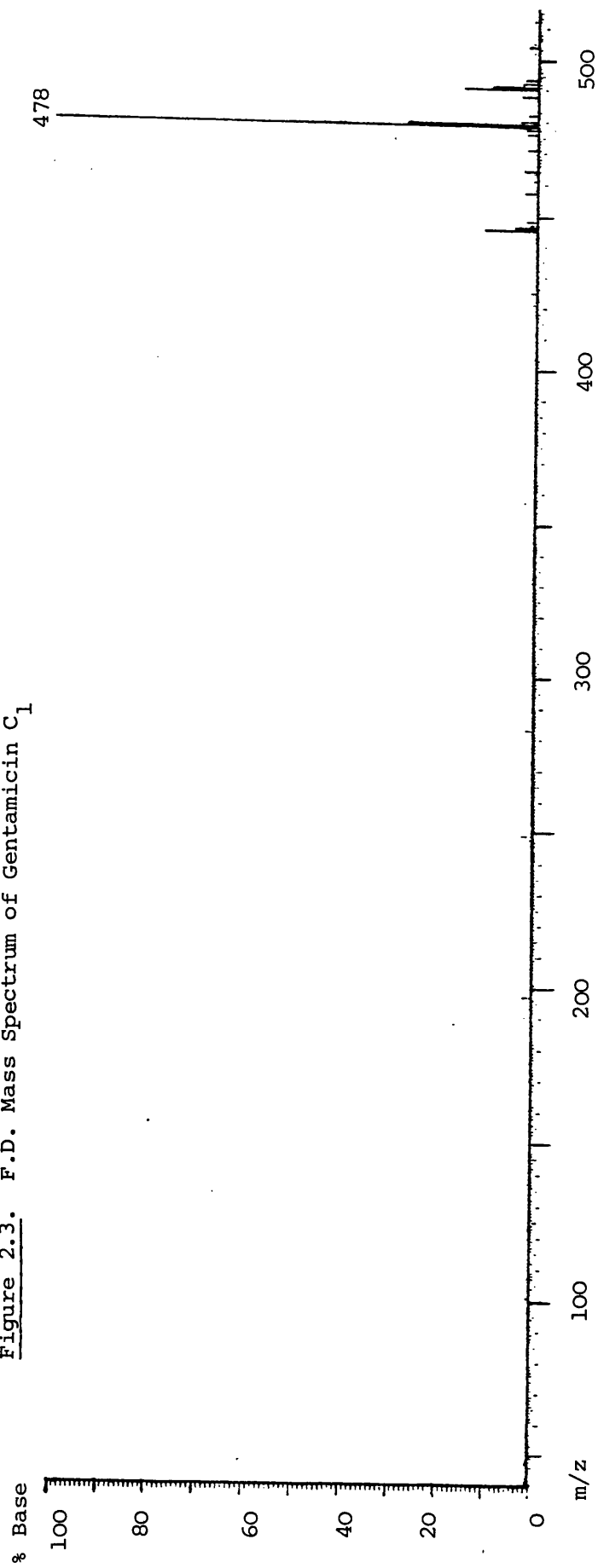


Figure 2.4. F.A.B. Mass Spectrum of Gentamicin C_{1a} ([+] 10N)

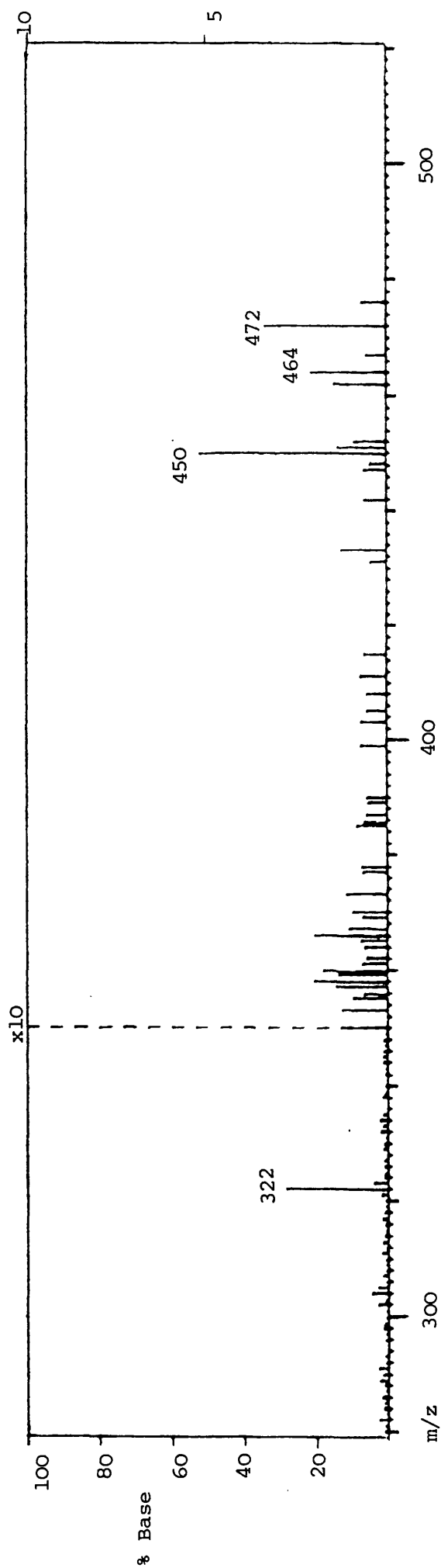
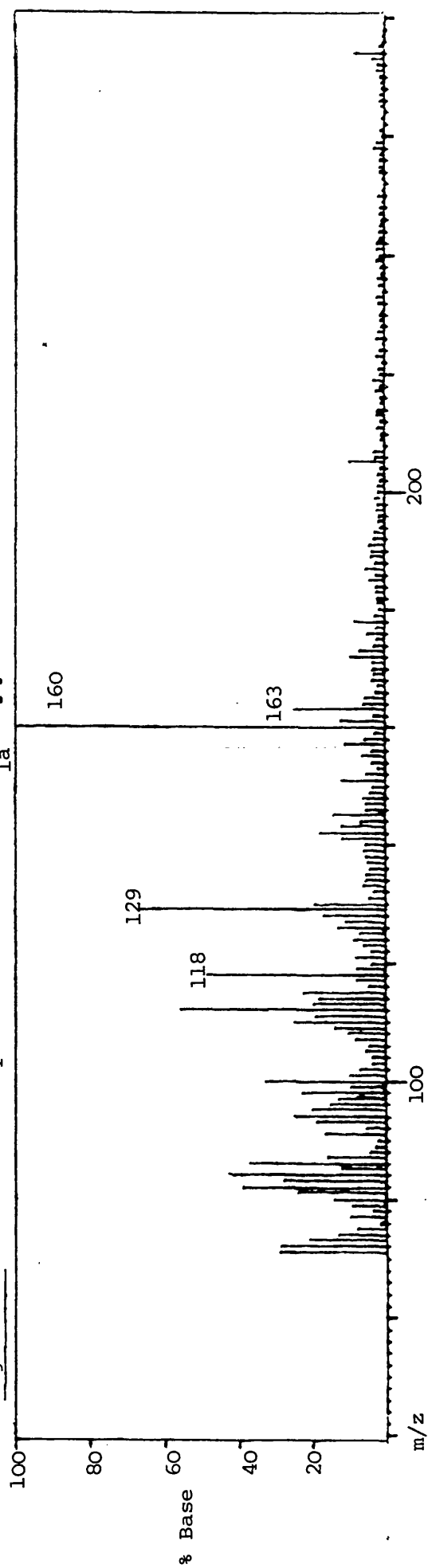
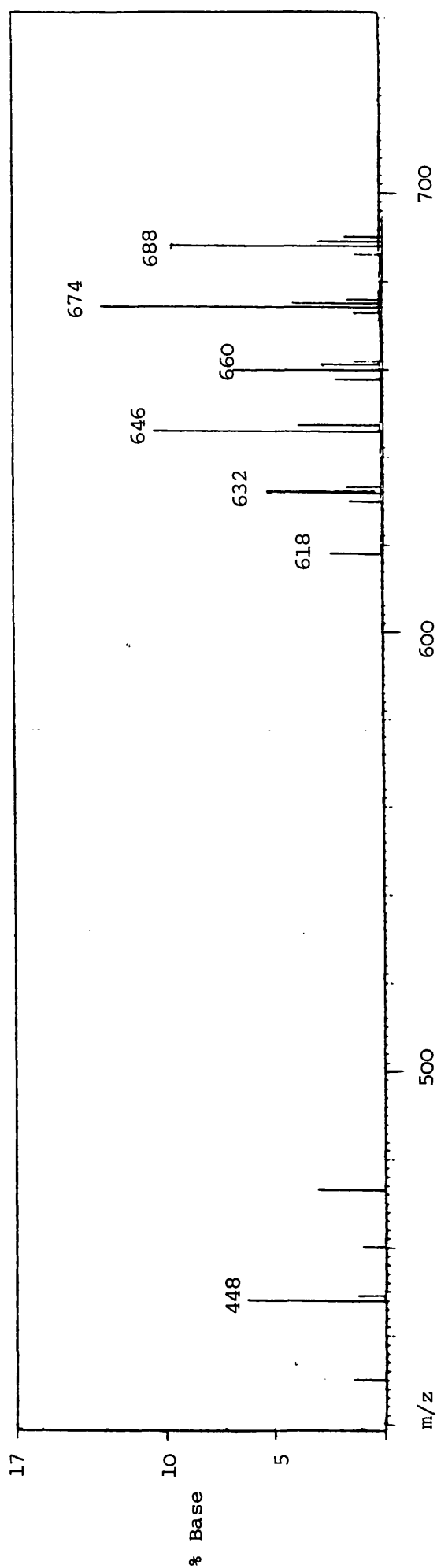
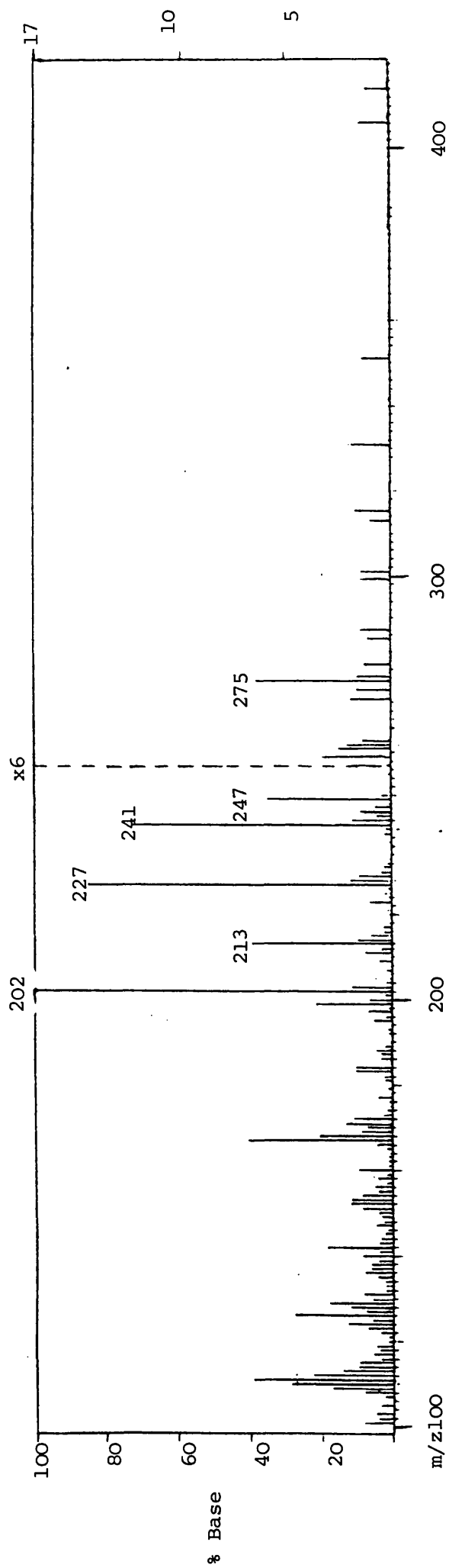


Figure 2.5. F.A.B. Mass Spectrum of N-Acetylated Gentamicin Components ($[+]$ ION)



Proton and Carbon Nuclear Magnetic Resonance Spectroscopy

Proton NMR of the gentamicin C components does not produce very distinctive results. A 100 MHz spectrum of gentamicin C₁ is illustrated in Figure 2.6. This shows one secondary methyl group at δ 1.04 ($J = 6$ Hz), one tertiary methyl group at δ 1.18 and two nitrogen substituted methyl groups at δ 2.32 and δ 2.51. The only other readily identifiable protons are the two anomeric protons appearing as overlapping doublets ($J = 4$ Hz for both) at about δ 5.1. The other components have spectra with slightly different patterns of methyl groups but no other discernible features. Hydrolysis of the gentamicins by acid produced 2-deoxystreptamine (2.I) which has the 100 MHz proton spectrum shown in Figure 2.7. The protons of C-2 have a shift of δ 1.30 for the axial and δ 1.96 for the equatorial proton. The $J_{aa} = 12$ Hz, $J_{ae} = 4$ Hz and the geminal constant was 13 Hz. The 1 and 3 hydrogens form a complex multiplet at δ 2.7 and the 4,5 and 6 hydrogens form the complex of multiplets at about δ 3.1. Further information on the spatial relationship of these protons was gained by Lemieux²⁶ who used methylation and acetylation of the functional groups to separate the protons.

Carbon-13 NMR of the major gentamicin C components shows virtually identical shifts for the garosamine and deoxystreptamine rings in all three²⁴⁶. The effect of the varying degree of methylation on the aminomethane sidechain at C-5' leads to slight but significant differences in the chemical shifts for the C-4' to C-6' carbons. The carbon-13 NMR spectrum

Figure 2.6. 100 MHz Proton NMR of Gentamicin C₁

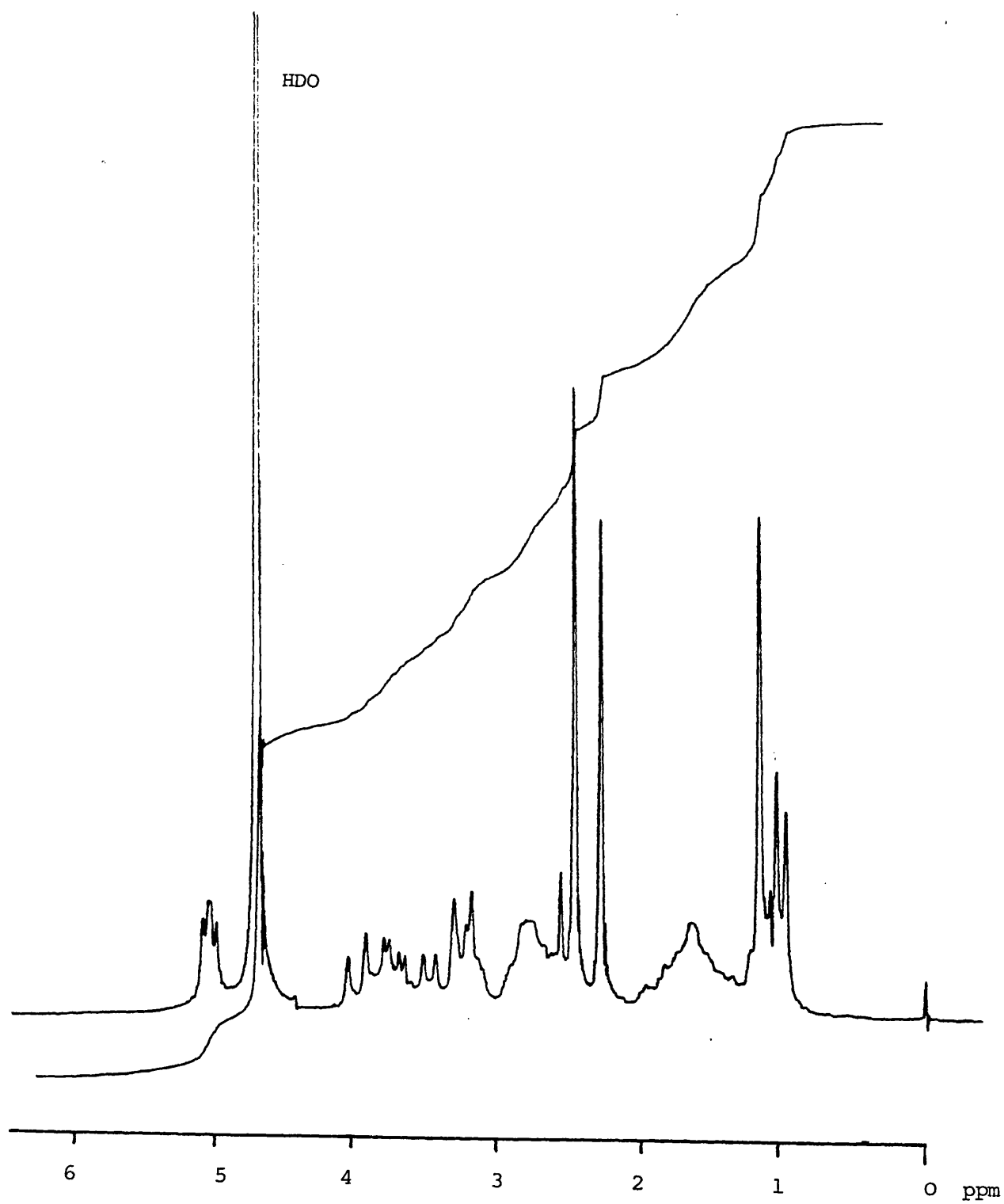
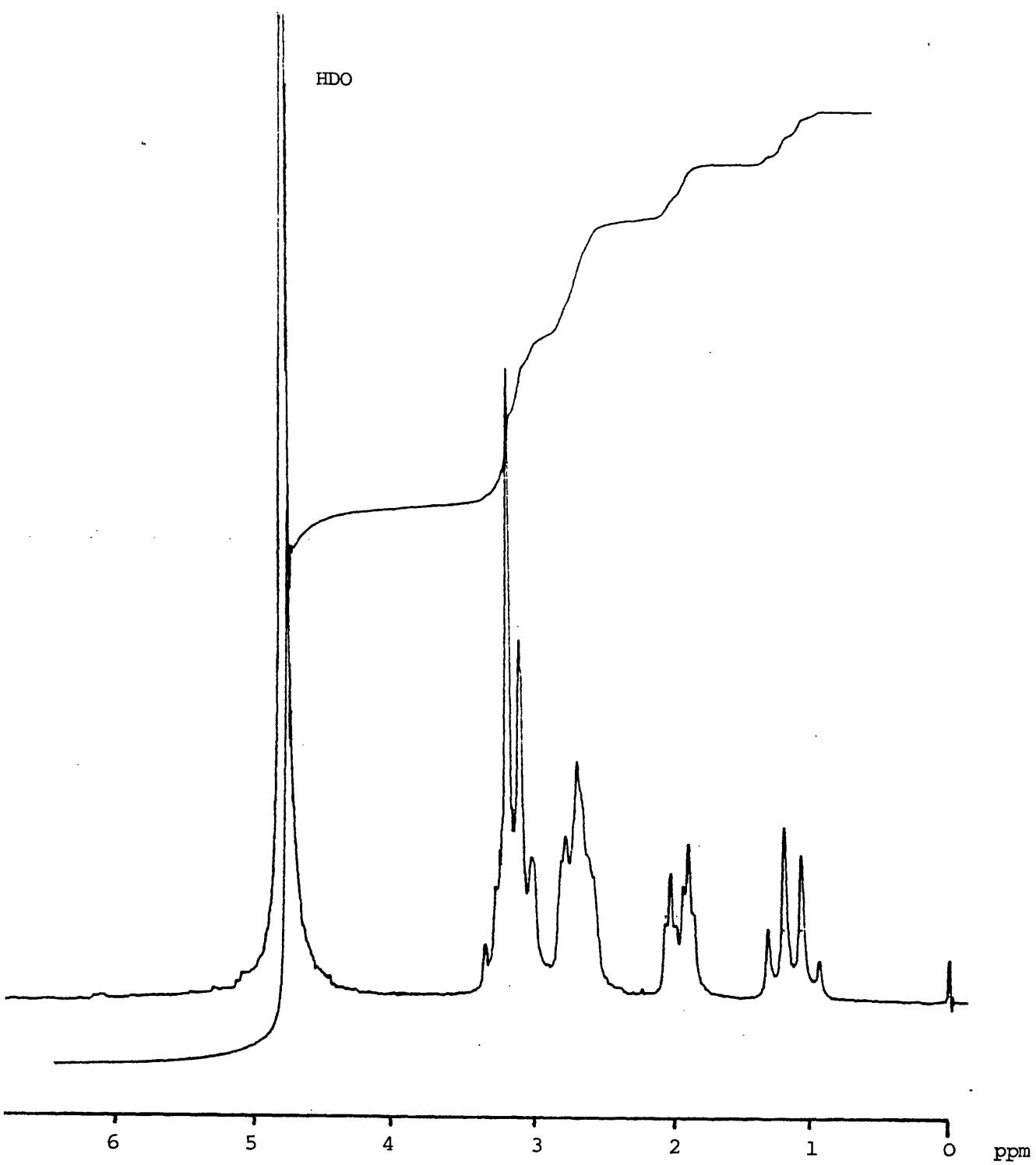


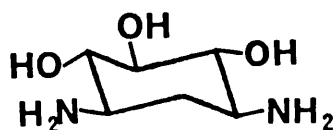
Figure 2.7. 100 MHz Proton NMR of Deoxystreptamine



of gentamicin C_1 is shown in Figure 2.8 with the chemical shifts denoted in Table 2.2. The chemical shifts of the carbon atoms of isolated deoxystreptamine are also given as are the shifts calculated for each carbon atom by the Lindeman and Adams equation²⁷³.

The chemical shifts reported here are similar to literature values²⁴⁶⁻⁷. The shifts for the deoxystreptamine moiety are comparable to those for the free compound and indicate the usefulness of spectral data of the individual sub-units. It is interesting to note that glycosidic bond formation at the C-4 and 6 does not have a large effect on the shift values of the remaining carbon atoms contrary to the calculated expectation. The calculations used are based on straight chain paraffins and extension of their use to cyclic systems increases the error. The shift values calculated for the anomeric carbons show significant error which must bear some relation to the glycosidic bond not exerting the expected electronegative pull.

It is noticeable that the C-5' and 5'' atoms have shift values also considerably less than predicted and this function is therefore inaccurately defined by the equation. Nevertheless, the use of these equations can prove useful in assigning peaks and investigating the effect of substitution of functional groups.



(2.I)

Figure 2.8. Carbon-13 NMR of Gentamicin C₁

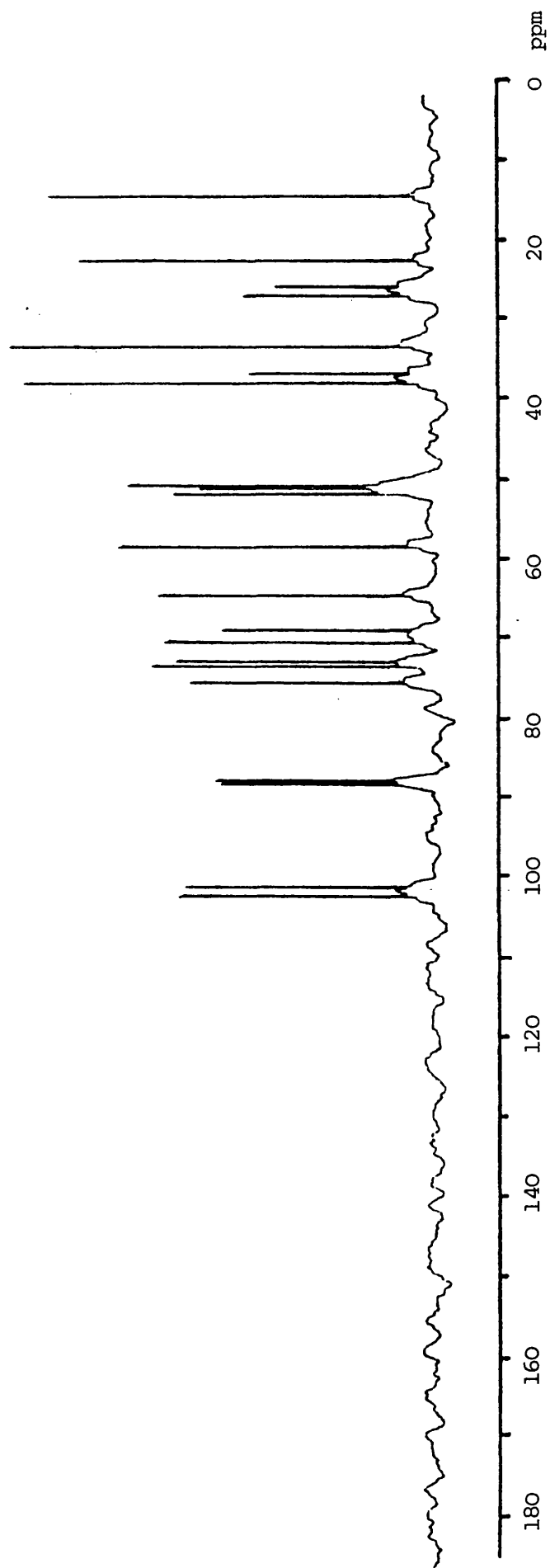
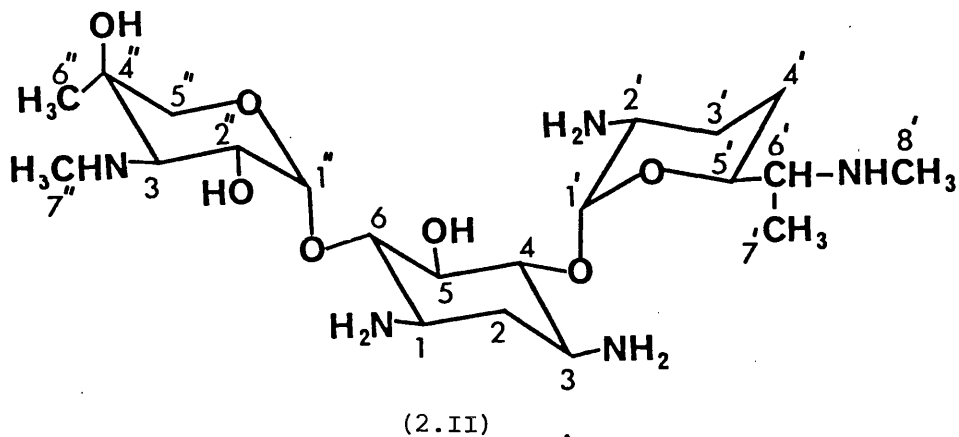


Table 2.2. Carbon-13 NMR shifts for gentamicin C₁ and deoxystreptamine

Carbon	Observed (ppm)	Calculated (ppm)	Observed (ppm)	Calculated (ppm)
1	51.91	46.92	51.90	51.92
2	36.92	38.98	37.49	40.98
3	50.82	46.92	51.90	51.92
4	88.59	88.53	79.09	81.53
5	75.73	71.46	77.25	77.46
6	87.63	88.53	79.09	81.53
1'	102.88	130.53		
2'	51.16	55.13		
3'	27.28	27.28		
4'	26.15	30.22		
5'	72.89	84.59		
6'	58.40	47.60		
7'	14.87	20.14		
8'	33.64	36.40		
1''	101.68	126.46		
2''	70.43	74.53		
3''	64.51	59.05		

/contd..

Table 2.2 continued

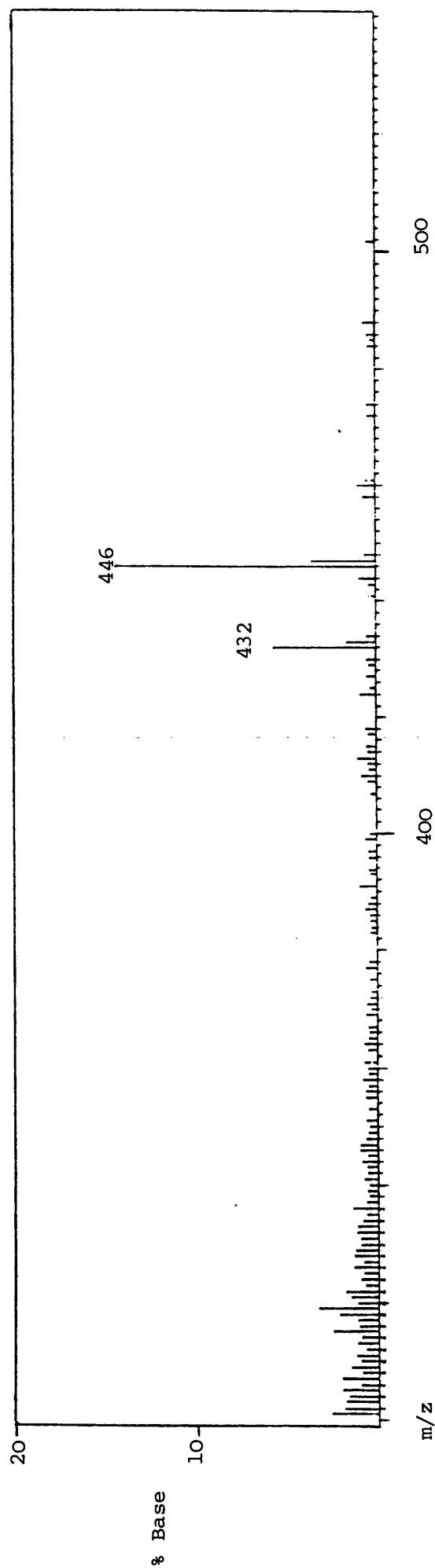
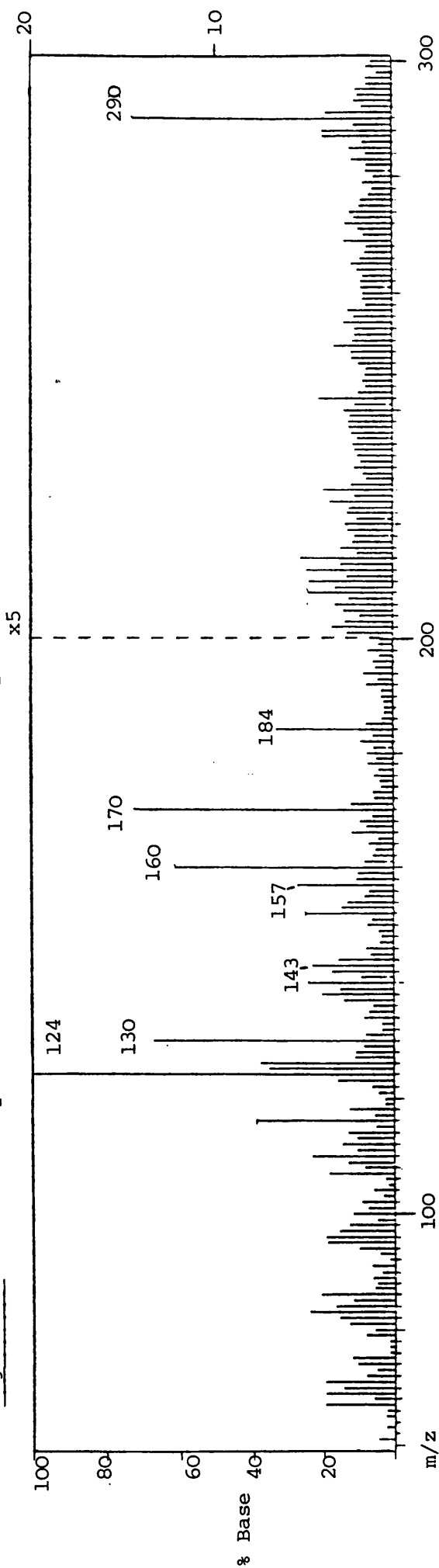
Carbon	Observed (ppm)	Calculated (ppm)
4"	73.52	68.51
5"	68.99	79.89
6"	22.87	25.29
7"	38.24	33.50

2.3.3 Isolation of Minor Components

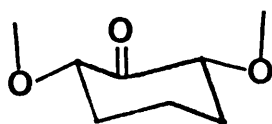
Swiss-Italian Samples:

Swiss (Benzian, BN/GO22, GMC-L-8) and Italian (Pierrel SpA, Lot 064/065) gentamicin sulphate samples were subjected to column chromatography. A total of 9.2 g of gentamicin base was separated on seven columns with a further two columns used to purify the isolated minor components. This second column yielded a fraction (54 - 75 ml) of about 2 mg which revealed two spots by TLC, with R_{C_1} values of 1.84 and 1.59. In this system the R_{C_1} of gentamicin C_2 and C_{1a} were 0.79 and 0.39 respectively. The isobutane CI spectrum of the minor components is shown in Figure 2.9. The peaks at m/z 446 and 432 are $[M+H]^+$ signals and the difference of 14 suggests they are structural homologues. The presence of a garosamine moiety is suggested by the peak at m/z 160, but there is no signal at m/z 163, 191 or 145. The absence of the characteristic peaks of m/z 350, 322 and 304 further verify the lack of a deoxystreptamine moiety. These components are thus similar to those discovered by Kraisintu⁷⁷. The prominent peak at m/z 290 is possibly due to a two ring fragment equivalent to the m/z 322 of the gentamicins. This implies a fragment of m/z 131 for the central ring if it behaves similarly to deoxystreptamine. The ring shows a large signal at m/z 130 which may correspond to the ring without a proton addition. The B ring is thus 32 mass units smaller than 2-deoxystreptamine and various structural interpretations can be proposed for this loss. The 'A' ring in these components is required to be 157 and 143 which are the purpurosamines A and B observed in gentamicins

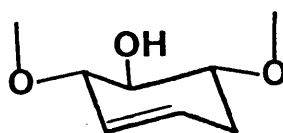
Figure 2.9. C.I. Mass Spectrum of Swiss/Italian Minor Components



C_1 and C_2 . These are in fact seen in Figure 2.9 and confirm the size of the B-ring. Further characterisation of these components was achieved by N-acetylation with the FAB spectrum illustrated in Figure 2.10. No clear molecular ions are visible, probably due to fragment combination. However, peaks corresponding to tri-N-acetylated compounds plus sodium occur at m/z 594 and 580 with no greater N-acetylated ions present. Garosamine is disclosed by the signal at m/z 202, with the purpurosamines at m/z 241 and 227. There is no peak at m/z 130 or at 172 or 214 (N-acetylated). It is possible that the m/z 130 fragment of the B ring has become associated with glycerol or some other species. The peak at m/z 304 is also present in the FAB spectrum of the unacetylated minor components but absent in the CI spectrum (Figure 2.9). It may represent a dimer of ring B and sodium ($129 + 23$). There is a m/z 152 peak present but this is comparatively small (8%) and there is a larger peak at m/z 168 which could be ring B plus potassium. It is therefore likely that the central moiety is not stable in the FAB conditions employed but there is no indication of N-acetylation. The uneven molecular weight of the components indicates an odd number of nitrogen atoms present. The overall evidence is that there are only three amino groups in the components and these are distributed in the A and C rings. The B ring with a weight of 128 and possible molecular formula of $C_6H_8O_3$ could have a general structure of either (2.III) or (2.IV)

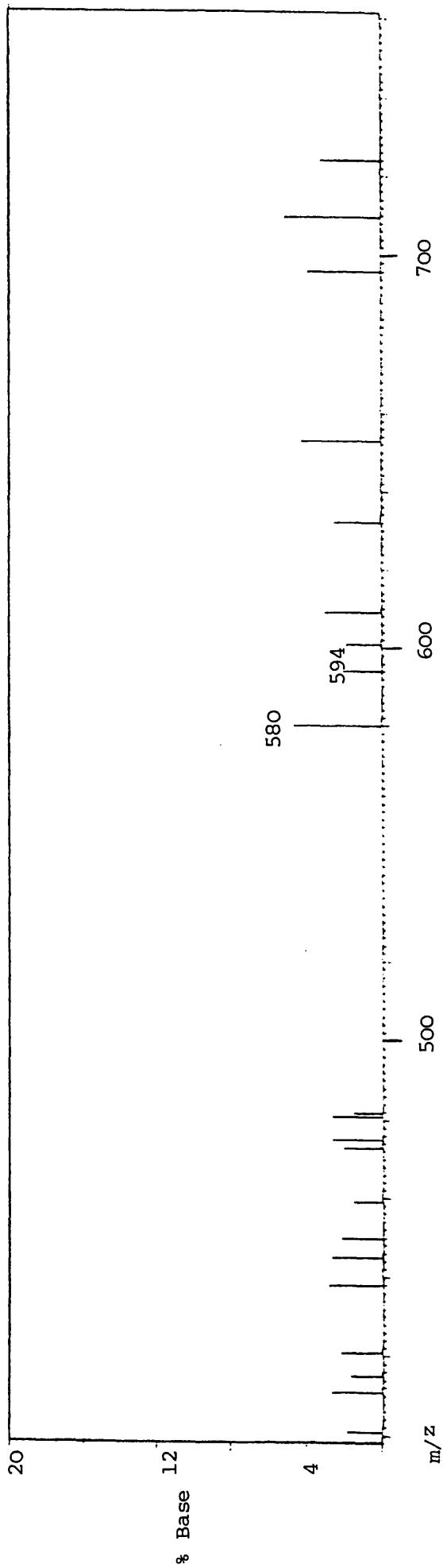
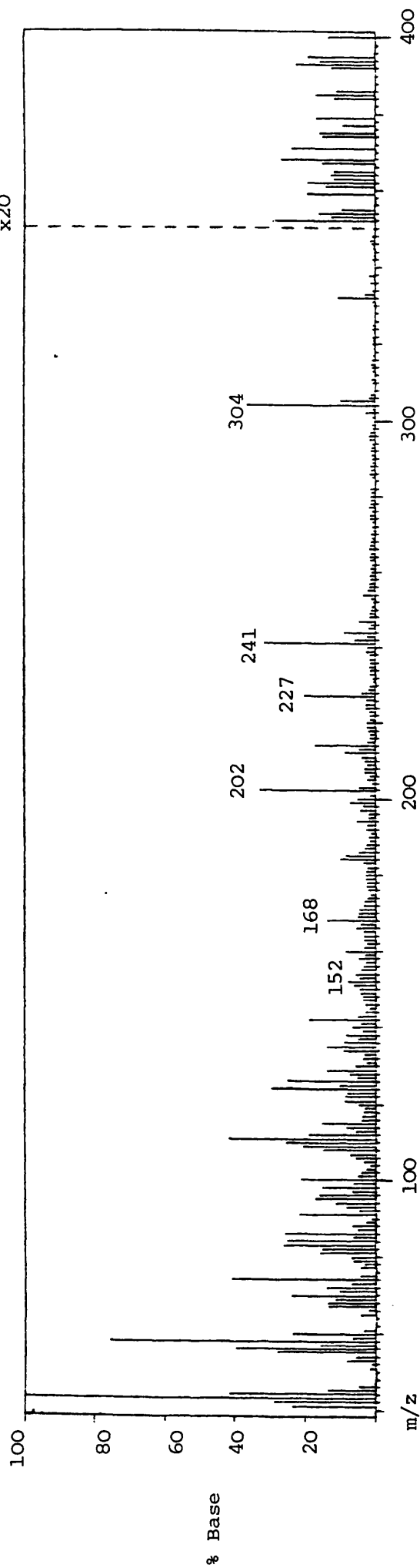


(2.III)



(2.IV)

Figure 2.10. F.A.B. Mass Spectrum of Swiss-Italian Minor Components - N-Acetylated ([+] ION)



More specific structural assignments would require additional sample to enable carbon-13, proton NMR and degradative studies to be performed. The components, however, appear to represent a family of gentamicins identical to the C-components, but without the deoxystreptamine ring. The microbiological activity of these components would be low according to Price⁴⁶ as they lack a basic function at the C-1 and 3 positions. However, Kraisintu⁷⁷ reported similar activity to the C-components. These minor components would be resistant to micro-organisms which produce the AAC-3 inactivating enzyme.

Italian Sample

A different batch of Pierrel (BN Genta/121) gentamicin base (26 g) was chromatographed on a column to isolate more of the 445/431 components. However mass spectral analysis and TLC was unable to detect them in appreciable yield. Some of the fractions of minor components were N-acetylated in an attempt to identify some of those present. The fractions of F551-90 and F591-630 show peaks at m/z 424 and 410 amongst the numerous signals present (Figure 2.11). N-acetylation of these fractions revealed peaks at m/z 452/466, 494/508 and 536/550 (Figure 2.12), which represent mono, di and tri-acetylated derivatives of the 423/409 components. Garosamine is again present as shown by the m/z 160 and 202 in the separate spectra. The N-acetylated spectrum indicates that purpurosamines A and B are present because of the sizeable m/z 227 and 241 signals. However the spectra also show that the components of m/z 445/431 are present

Figure 2.11 FAB Mass Spectrum of Italian Minor Components ([+] ION)

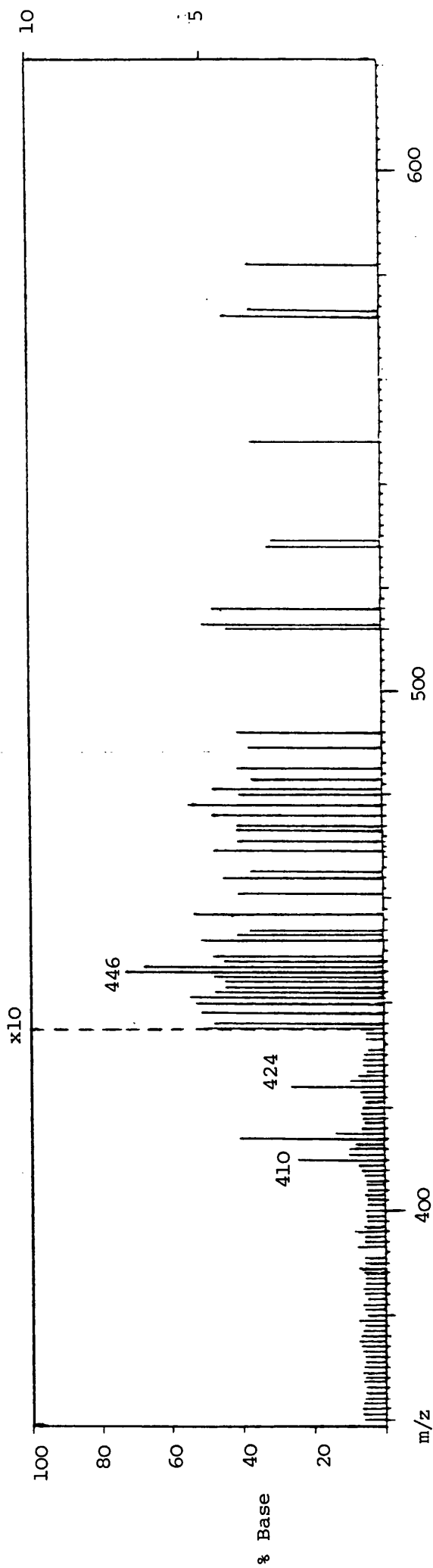
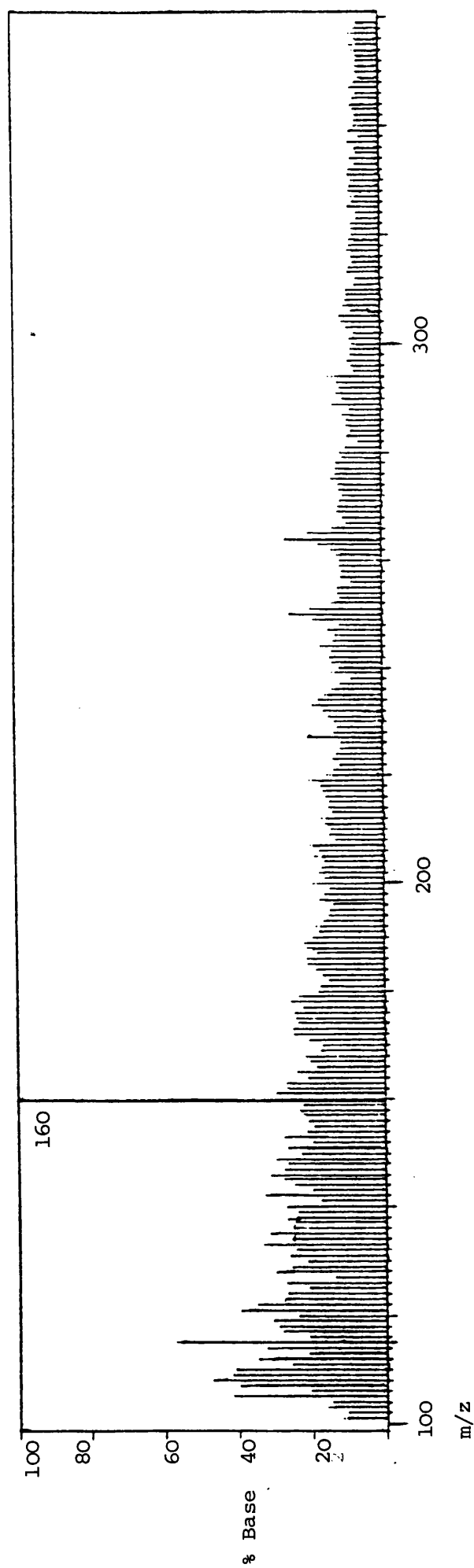
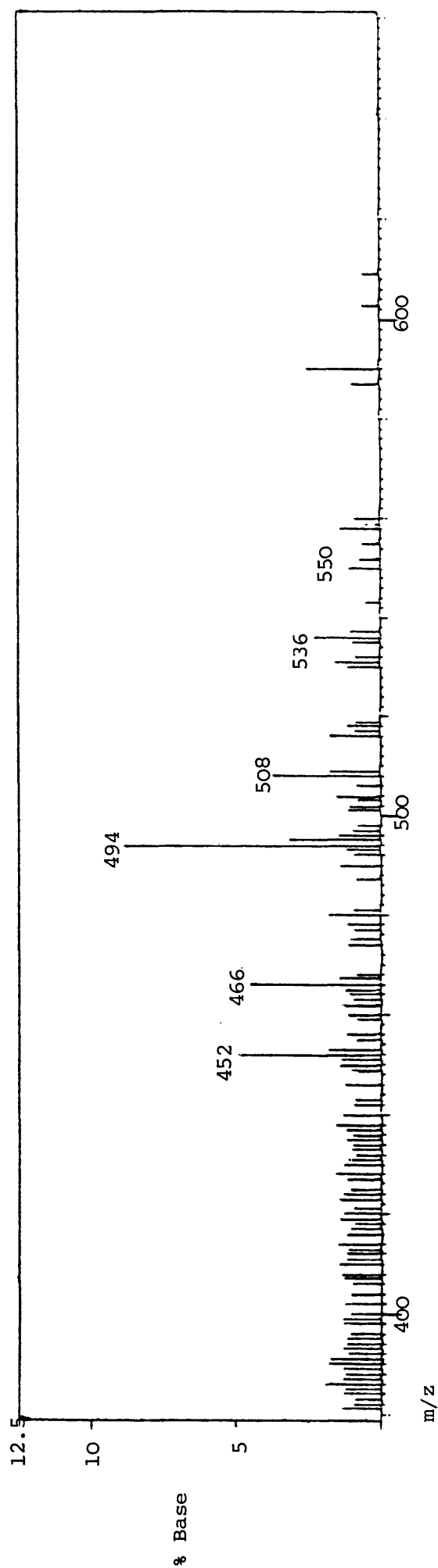
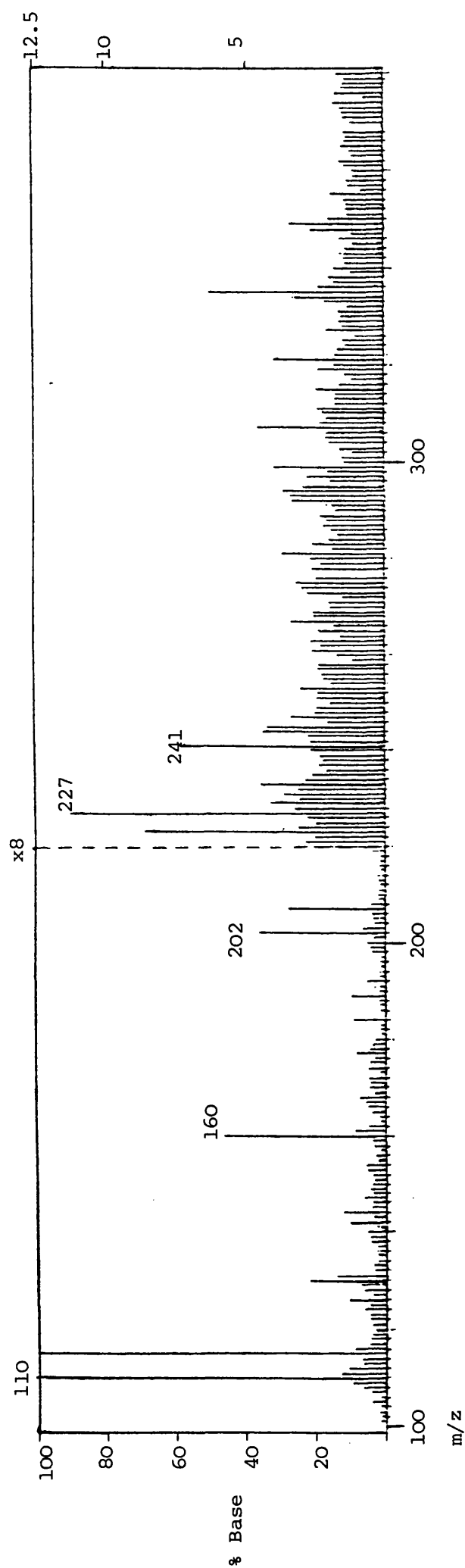


Figure 2.12. FAB Mass Spectrum of Italian Minor Components - N-Acetylated ([+] ION)



in the fraction and peaks due to purpurosamines may originate from them. No other explainable peaks are observed for the ring A and if these components do contain the purpurosamines the central ring must possess a molecular weight of only 106. A peak at m/z 108 is present but is small and addition reactions may have occurred. Thus analysis of gentamicin sulphate samples from the Italian manufacturer yielded small quantities of potentially interesting components with major changes of the central sub-unit.

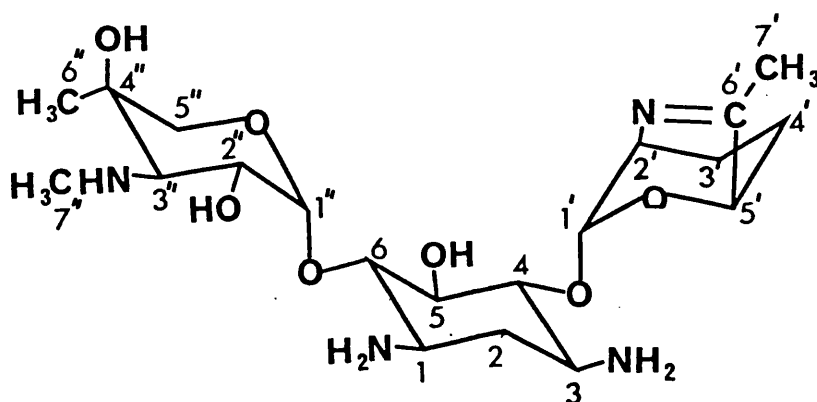
Chinese Sample:

A sample of gentamicin sulphate of undetermined source, but thought to be Chinese was chromatographed (15.2 g) on four columns. Unlike the gentamicin samples studied in a) and b) the minor components eluted as clear distinct zones and were thus more amenable for isolation. The profile of minor components eluting before gentamicin C_1 was as shown in Table 2.3 with R_{C_1} values given.

Table 2.3. Minor component profile of Chinese material

Component	Weight (mg)	R_{C_1}
CM1	< 1	3.52
CM2	2	2.39
CM3	< 1	1.99
CM4	80	1.70
CM5	50	1.31

The CM1 and CM3 components were not isolated in sufficient yield to achieve any identification. The field desorption, chemical ionization and FAB spectra all depicted m/z 445 as the $[M+H]^+$ ion. The EI spectrum of CM4 is illustrated in Figure 2.13. Peaks at m/z 350, 322, 191, 163 and 160 all imply that garosamine and deoxystreptamine are present as the B and C rings. The remaining A ring has, by subtraction, to be a fragment of m/z 124 and possess an odd number of nitrogen atoms. There is indeed a peak of m/z 124 present but this is not conclusive as garosamine can lose water to produce an ion of this mass. However the peak at m/z 286 is indicative of a 124 substituent joined to deoxystreptamine and it is not usually found in gentamicin spectra (Figure 2.2). Bérdy⁷⁴⁻⁷⁵ isolated a compound of the same molecular weight and assigned a structure (2.V) to it although he offered no supporting evidence.



(2.V)

The 100 MHz proton NMR spectrum of CM4 is presented in Figure 2.14 which can be compared with a similar spectrum of gentamicin C₁ in Figure 2.6. The single methyl at δ 1.20

Figure 2.13. E.I. Mass Spectrum of CM4

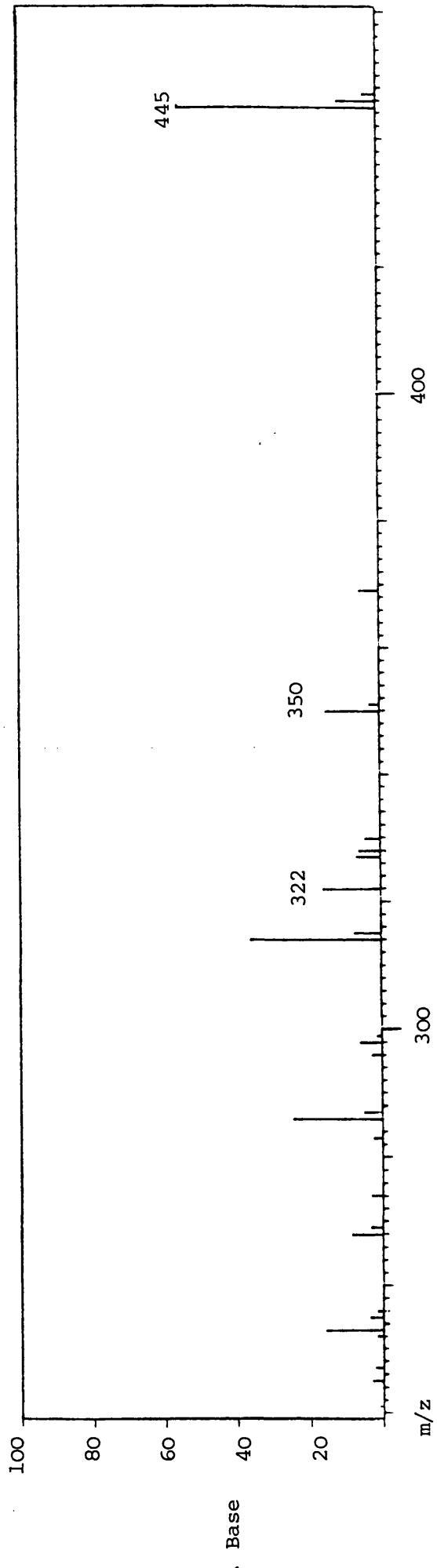
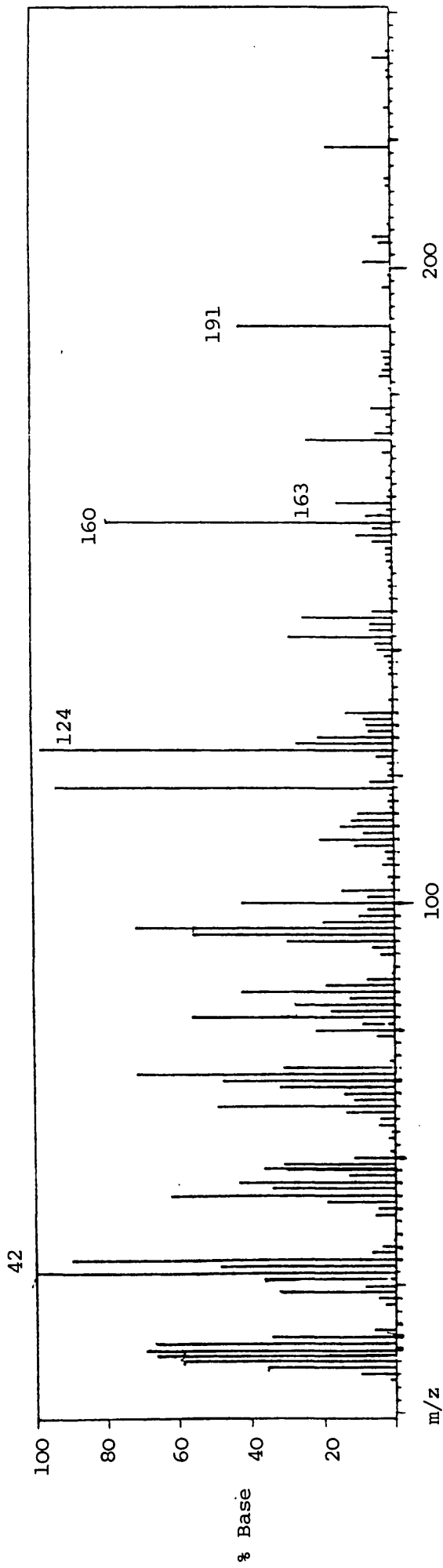
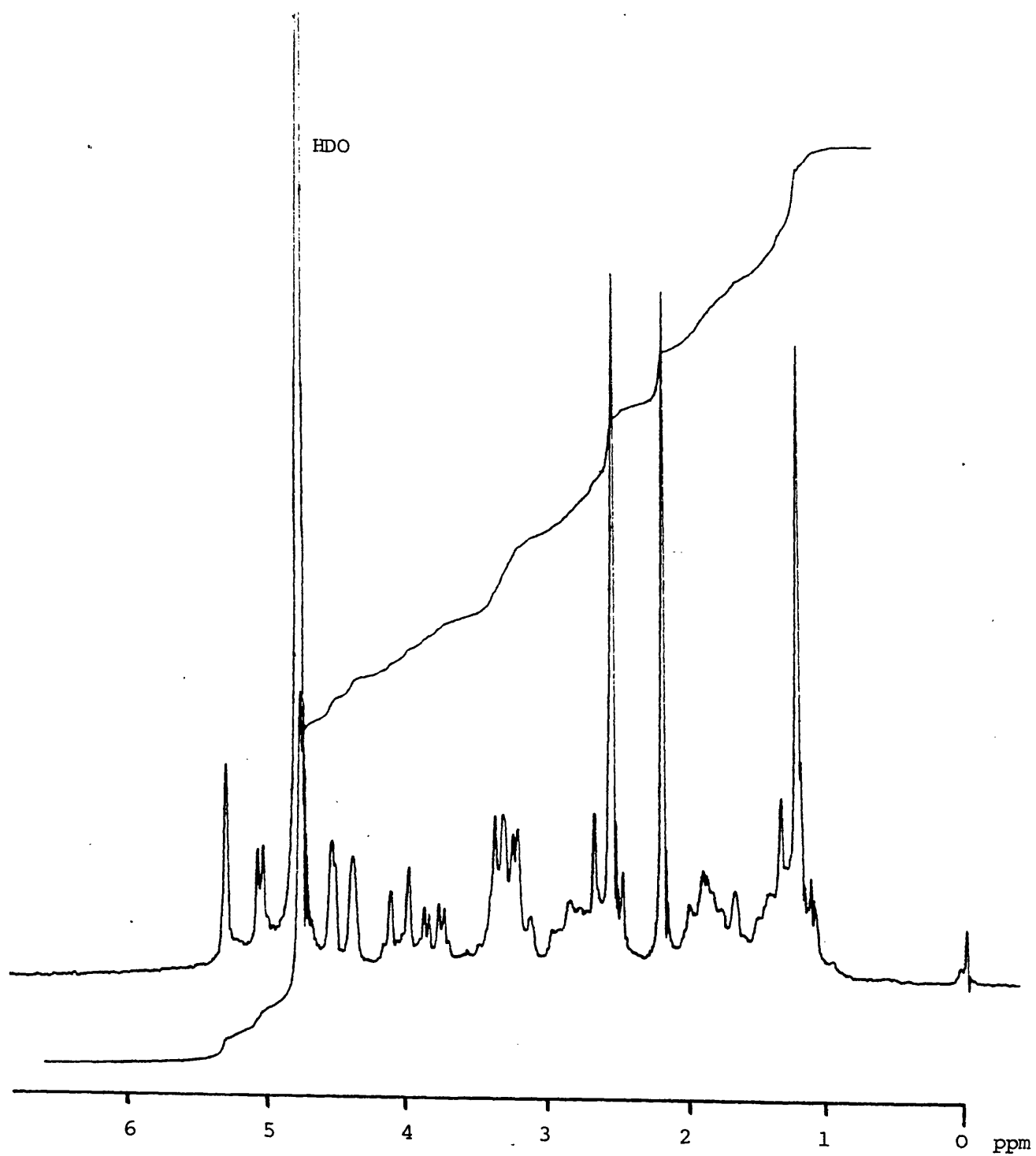


Figure 2.14. 100 MHz Proton NMR Spectrum of CM4



represents the tertiary methyl at C-4" and compares to δ 1.18 for gentamicin C₁.

No secondary methyl group is evident but two methyls appear in the δ 2-3 region. The peak at δ 2.53 is comparable to δ 2.50 for gentamicin C₁ and represents the amino substituted methyl in the garosamine ring. The other peak at δ 2.16 does not correspond closely with the δ 2.32 found for the aminomethyl group on C-6' in gentamicin C₁. It could plausibly represent a methyl group adjacent to an imine function but could also fit other groups. One of the anomeric protons is shifted to δ 5.26 and broad peaks appear at δ 4.35 and 4.51. The latter may be the C-2' and C-5' deshielded protons.

The carbon-13 NMR spectrum of this compound is reproduced in Figure 2.15 and assignments of the shifts are made in Table 2.4, with a comparison to gentamicin C₂. Two peaks representing carbons 3' and 5' were not detected even after a duplicate run. Otherwise the resonances were very similar to those reported by Kraisintu⁷⁷. The shifts of the B and C rings show a close comparison to those found in gentamicin C₂ confirming mass spectral analysis. For the A-ring, the C-2' is deshielded by 5.5 ppm and the peak of 181.96 is the carbon of the imine function. This is similar to some literature values of imines and lies outside the normal aldehyde/ketone region²⁷³⁻⁴. The shift of the methyl group (C-7') attached to the C-6' is anomalous since the presence of the imine function would be expected to deshield greater than

Figure 2.15: Carbon-13 NMR Spectrum of CM4

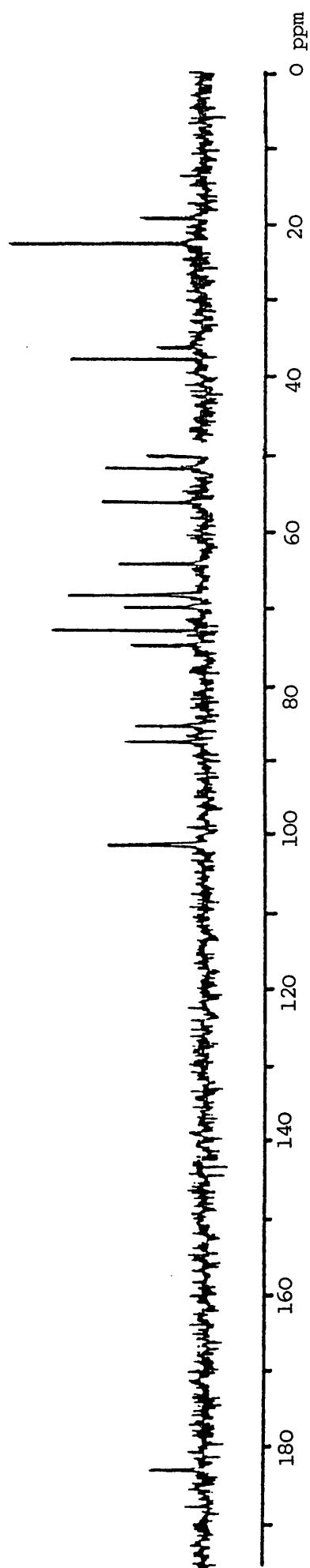


Table 2.4. Carbon-13 NMR shifts of CM4 and Gentamicin C₂

Carbon	CM4 (ppm)	Gentamicin C ₂ (ppm)
1	51.50	51.34
2	35.90	36.07
3	49.83	50.37
4	87.58	88.34
5	74.69	75.07
6	85.53	87.42
1'	101.02	102.43
2'	56.11	50.63
3'	-	26.56
4'	24.47	24.47
5'	-	74.15
6'	181.96	49.83
7'	18.89	18.51
1''	101.24	101.02
2''	69.82	69.98
3''	64.13	64.02
4''	72.80	72.96
5''	68.40	68.35
6''	22.31	22.37
7''	37.37	37.53

0.3 ppm, especially considering the proposed shift in the proton spectrum (Figure 2.14). Thus the spectroscopic data suggests, but is not adequate to confirm, structure (2.V).

In order to confirm the presence of an imine group the compound was reduced with dimethylamine borane²⁷⁵. This reagent, will reduce an imine to the amine and carbonyl to the alcohol,²⁷⁶⁻²⁷⁷ and was chosen so that the product could be easily isolated. The actual reduction can be judged to occur by comparison of the infra-red spectrum of the CM4 component with that of the reduced product shown in Figure 2.16. A weak band is visible at 1640 cm^{-1} on the broader band at 1600 cm^{-1} due to the N-H bending. This is expected for a C=N- group and values of $1665\text{--}1645\text{ cm}^{-1}$ of variable bond strength are quoted²⁷⁸ for compounds of the type R-C=N-R . Carbonyl absorption bands tend to be strong and higher in frequency, This band at 1640 cm^{-1} is absent from the IR spectrum of the reduced component. The success of the reduction was confirmed by a FAB spectrum which showed a ratio of 4:1 of m/z 447 to 445. A peak at m/z 126, with little m/z 124 present confirmed that the site of reduction was in the purpurosamine ring and that m/z 124 in Figure 2.13 was due to this ring and did not arise from loss of water from garosamine. It is concluded that the structure (2.V) is probably correct and that the ambiguous NMR data was due to an insufficiently large sample size.

The CM2 component, only separated in a very small quantity, produced a $[\text{M+H}]^+$ ION OF m/z 459 in a field desorption mass

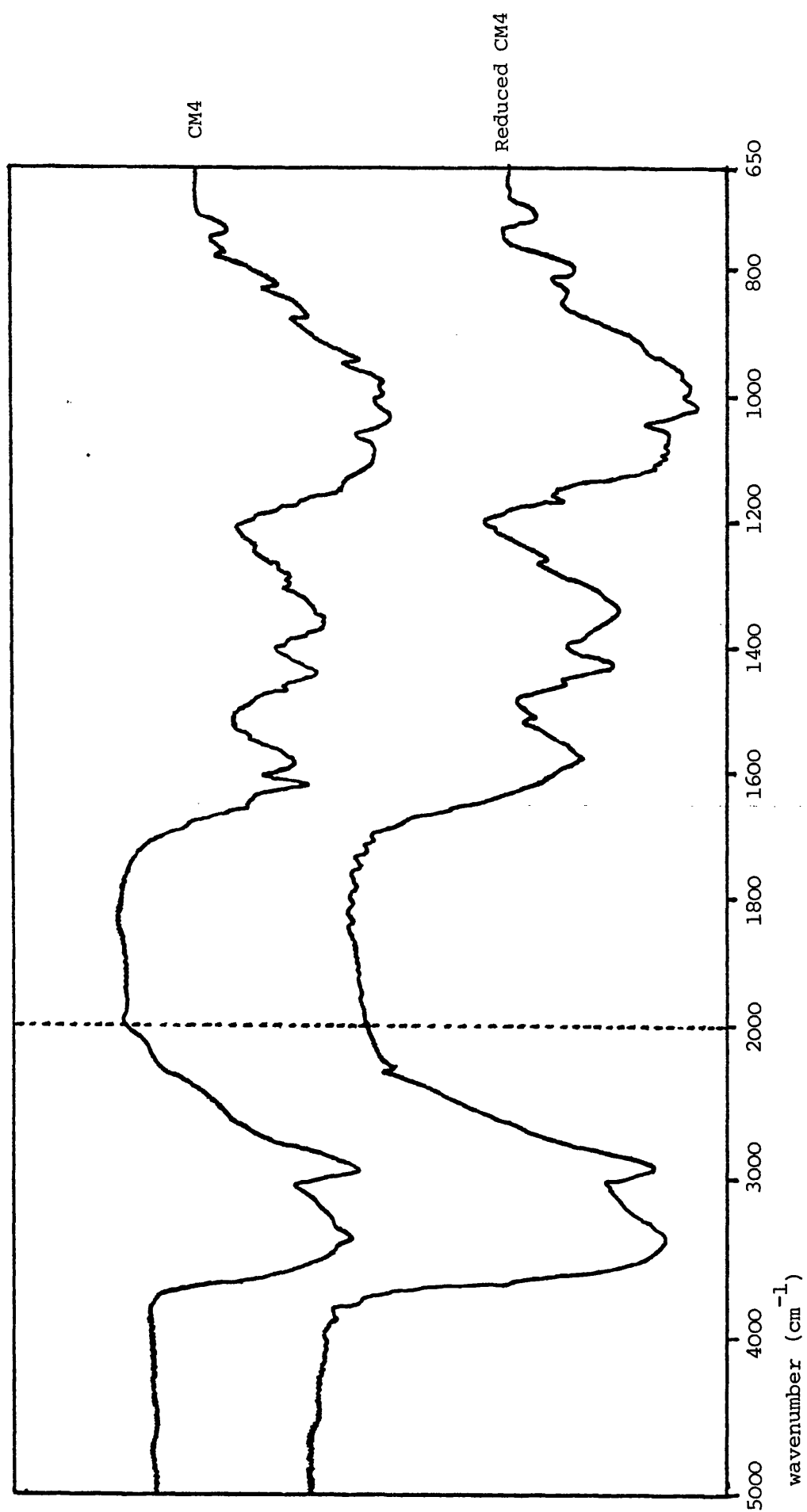
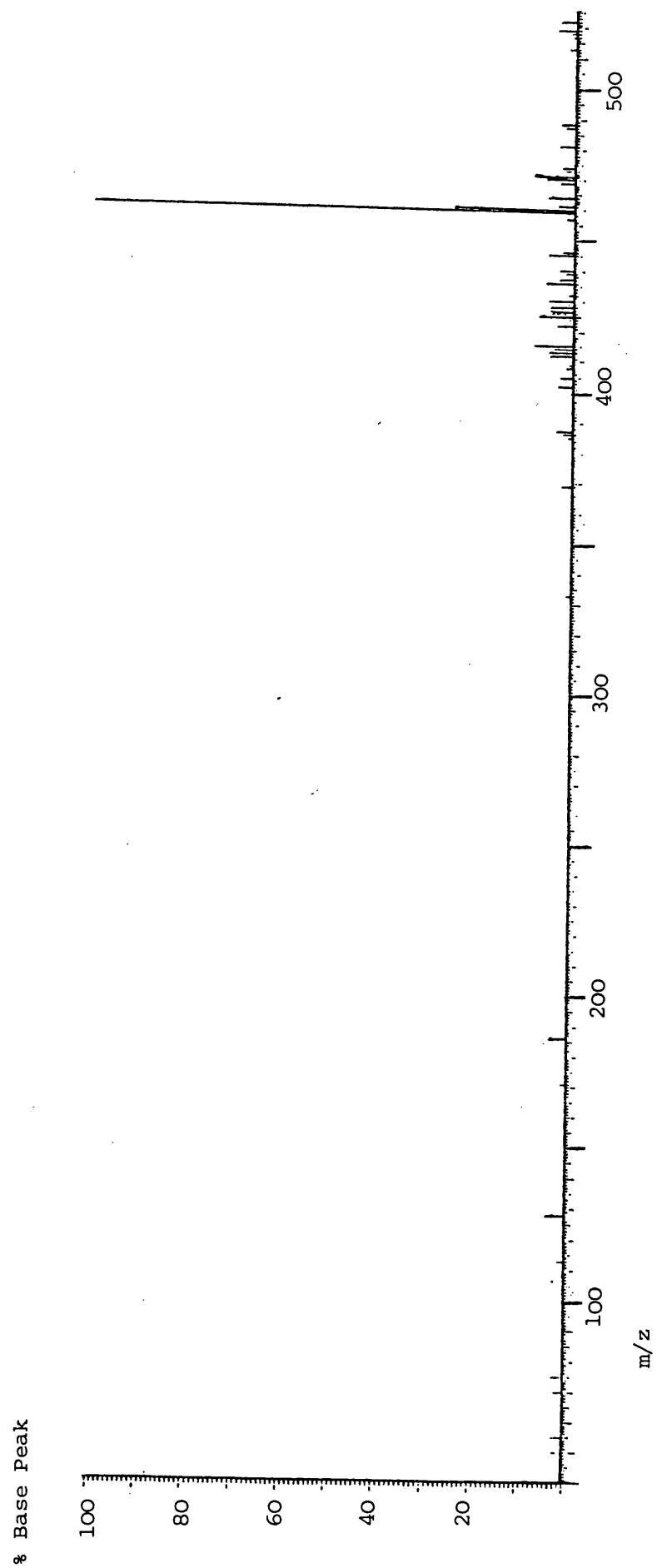


Figure 2.16. Infra-Red Spectrum of CM4 and Reduced CM4.

Figure 2.17. F.D. Mass Spectrum of CM2



spectrum (Figure 2.17). This is fourteen mass units larger than CM4 and is possibly a homologue. However the proposed structure of the A-ring excludes the normal methylation witnessed in the C components. Chemical ionization and FAB spectra of this component did not produce conclusive grounds for structural determination.

The CM5 component was procured in a significant yield. The field desorption mass spectrum of this compound indicated a molecular weight of 477, equivalent to gentamicin C_1 . The proton NMR spectrum of the component showed four methyl groups at shifts of δ 2.53, 2.35, 1.20, 1.08 ppm compared to shifts of δ 2.51, 2.32, 1.18 and 1.04 ppm for gentamicin C_1 . A carbon-13 NMR of the component was recorded shown in Figure 2.18, although one carbon atom could not be detected and splitting of other resonances also occurred. A comparison of chemical shift assignments with gentamicin C_1 is made in Table 2.5. There is little evidence of difference except for the splitting which mainly occurs in the A-ring. This was thought to be due to a mixture of stereo-isomers about the C-6' position. An important difference between the spectra is the peak attributed to the C-7' methyl which is deshielded by nearly 4 ppm and is of comparable size to the C-7' in gentamicin C_2 (18.51). This conflicting data was assessed by a CI spectrum of CM5 shown in Figure 2.19. The molecular weight is confirmed as 477, and garosamine is present. The signal at m/z 143 suggests that it is purpurosamine B and not A which is a sub-unit of the component. All signs of deoxystreptamine were

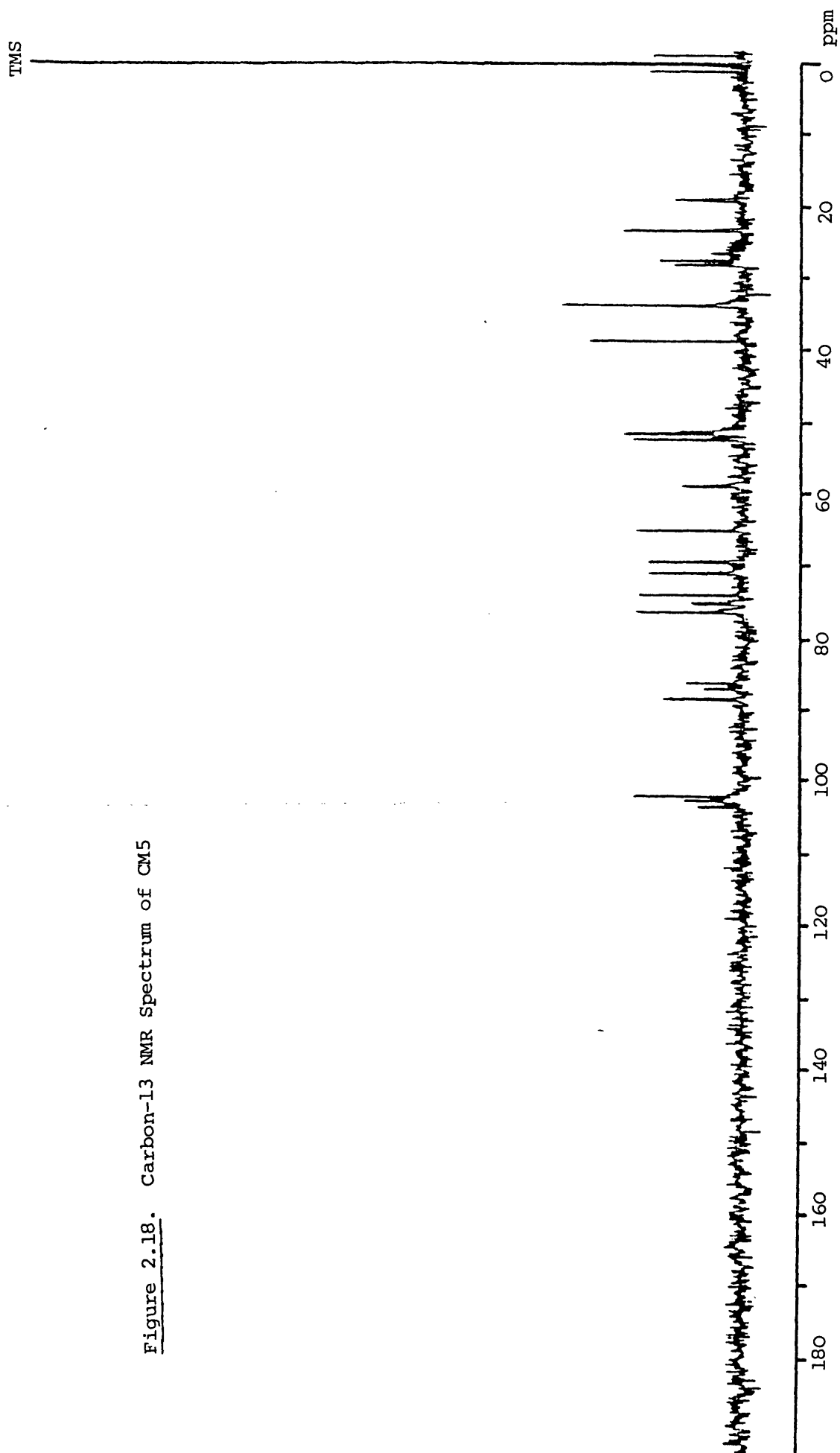
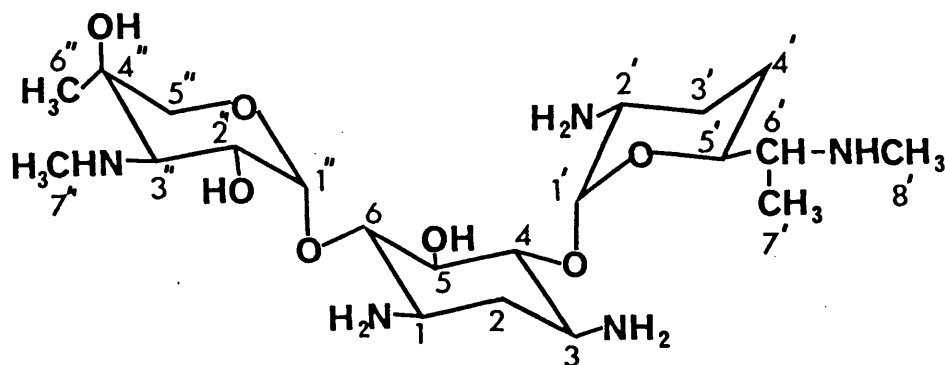
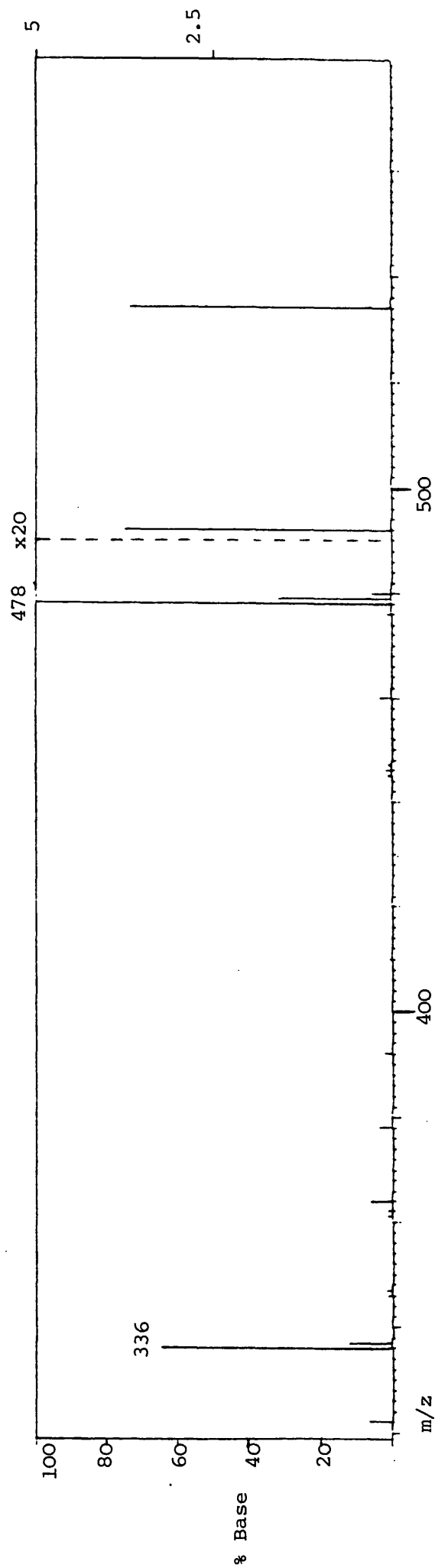
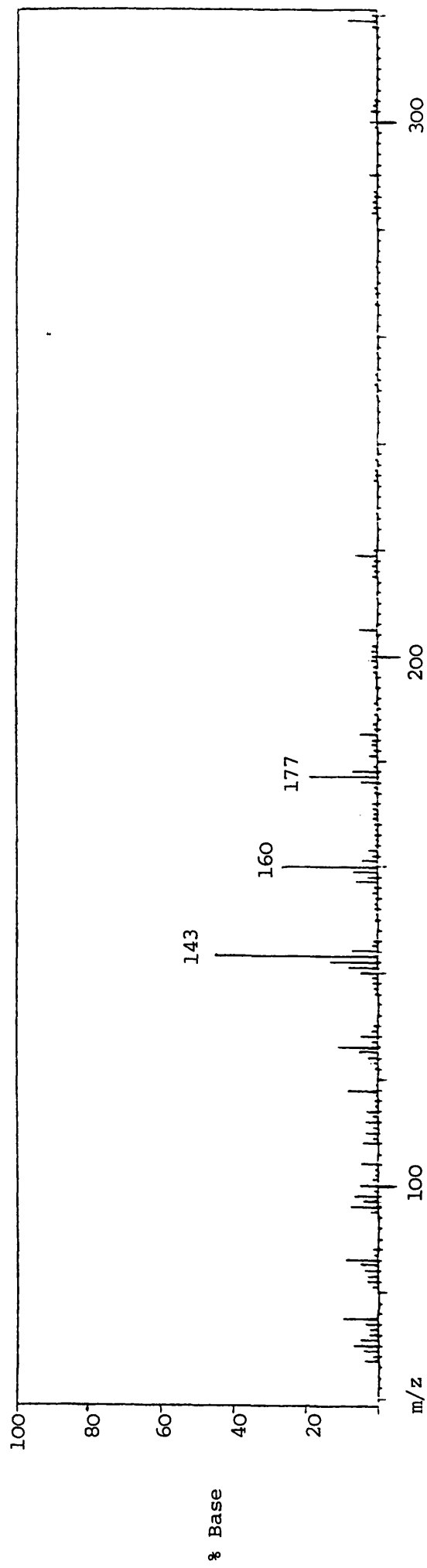


Figure 2.18. Carbon-13 NMR Spectrum of CM5

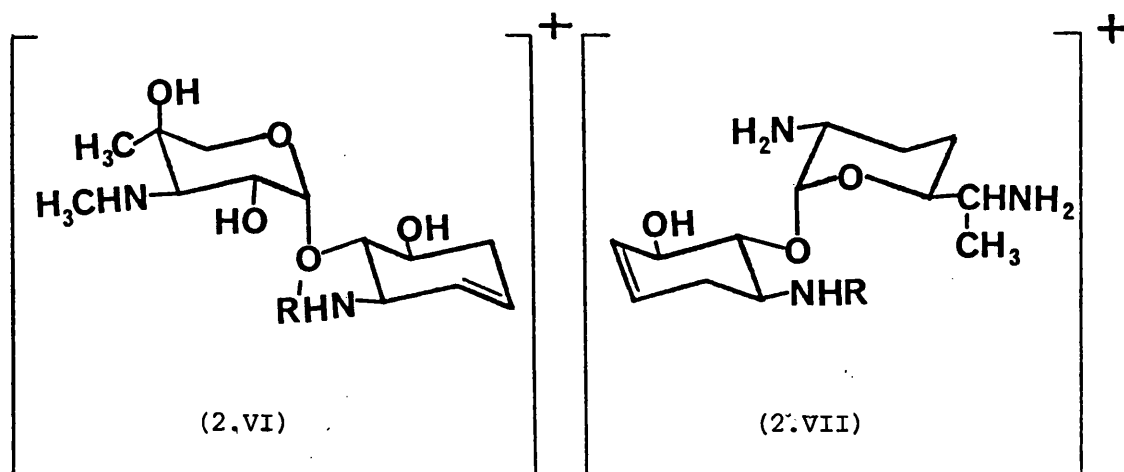
Table 2.5. Carbon-13 NMR shifts of CM5 and Gentamicin C₁

Carbon	Gentamicin C ₁ (ppm)	CM5 (ppm)
1	51.91	51.76
2	36.92	-
3	50.84	50.63
4	88.59	88.03
5	75.73	75.94
6	87.63	85.77/86.03
1'	102.88	102.26/103.14
2'	51.16	51.13
3'	27.28	27.64
4'	26.15	26.00/26.95
5'	72.89	74.56/74.87
6'	58.40	58.37
7'	14.87	18.64
8'	33.64	33.19
1''	101.68	101.63
2''	70.43	70.59
3''	64.51	64.80
4''	73.52	73.49
5''	68.99	69.01
6''	22.87	23.04
7''	38.24	38.16

Figure 2.19. C.I. Mass Spectrum of CM5

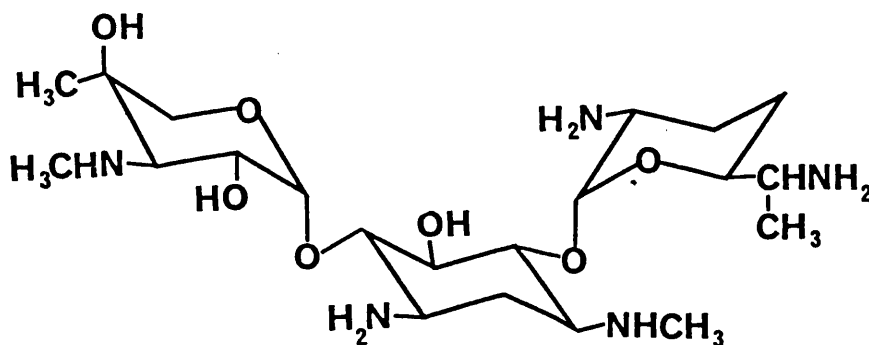


absent, but a strong peak at m/z 177 suggests N-methylation of deoxystreptamine. A peak at m/z 336 is equivalent to the m/z 322 characteristic peak of the gentamicins. Thus the CM5 component must be N-methylgentamicin C_2 . The carbon-13 NMR assignments given in Table 2.5 are therefore not accurate, as the shift for C-6' has to be switched with either C-1 or C-3 depending on the position of N-methylation and C-8' becomes C-7. The CI spectrum of CM5 was supported by a FAB spectrum of the N-acetyl derivative which had major peaks of m/z 688, 261, 227 and 202 indicating N-acetylated garosamine, purpurosamine B and N methyl deoxystreptamine. To determine the position of the N-methylation reference was made to a paper by Wright²⁰. A method using high energy EI mass spectrometry had been developed which related a fragmentation pattern to the site of N-alkylation in the deoxystreptamine ring. This pathway involved the loss of the amino group adjacent to the glycoside bond cleaved to produce two possible ions (2.VI, 2.VII)²⁰.



			(2.VI)	(2.VII)
$R^1 = CH_3$	$R = H$	1-N-Methylgentamicin C_2	m/z 303	272
$R^1 = H$	$R = CH_3$	3-N-Methylgentamicin C_2	m/z 289	286

Thus depending on which pair of ions is present, the site of N-alkylation can be determined. The 70 eV EI spectrum of CM5 is illustrated in Figure 2.20. The peaks of 289 and 286 are obvious whilst the alternative pair are not present. Thus CM5 must be 3-N-methylgentamicin C_2 and has the structure (2.VIII)

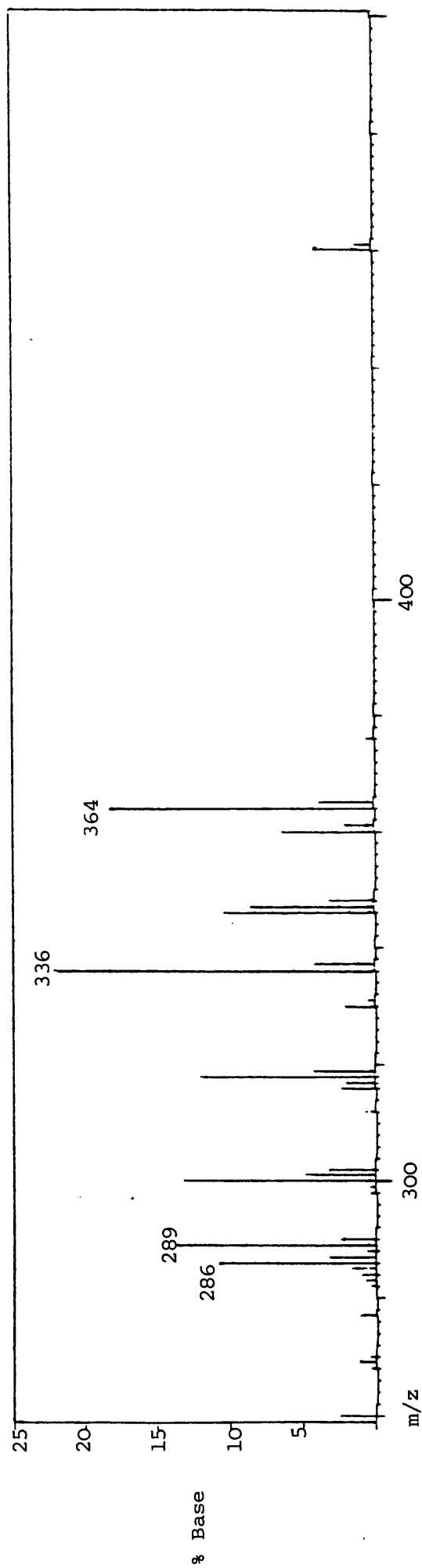
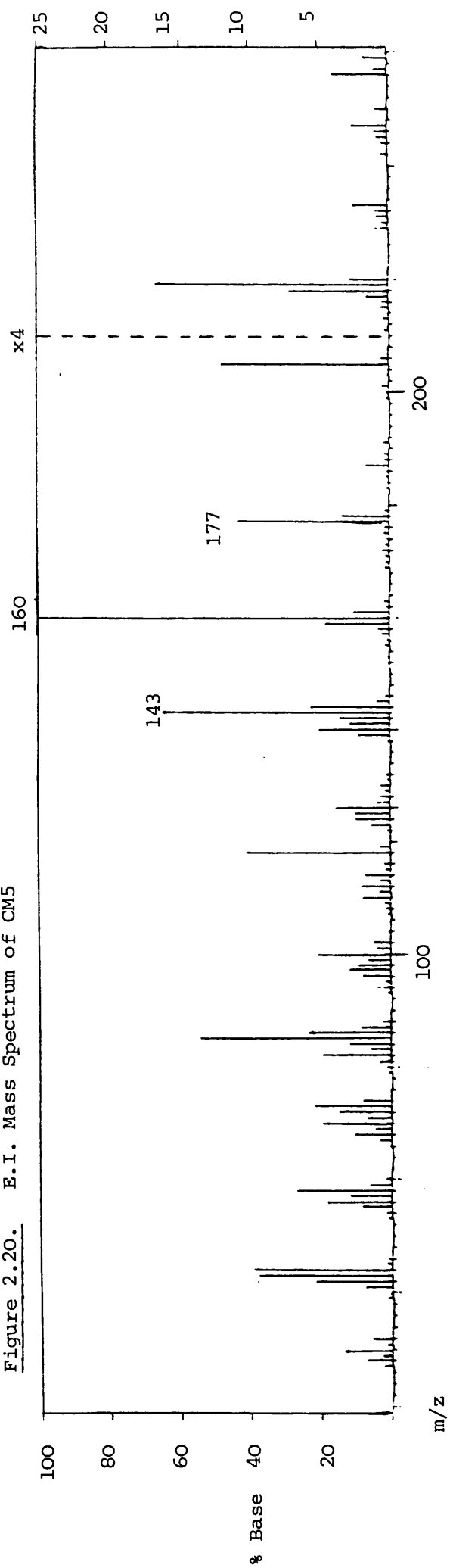


(2.VIII)

The position of N-methylation is important, as Wright²⁰ reported that the synthetic 3-N-ethylgentamicin C_{1a} derivative possessed weak anti-bacterial activity, whilst the 1-N-ethyl analogue possessed strong activity. If the CM5 component was active it would have an inherent resistance to AAC-3 enzymes, an important source of gentamicin inactivation.

The identification of CM5 can also be applied to the CM2 component discussed previously. It is possible that this component could bear a similar relationship to CM4 as CM5 does to gentamicin C_2 . N-methylation of the deoxystreptamine would leave the components with the same A-ring and would be consistent with the homologue idea.

Figure 2.20. E.I. Mass Spectrum of CM5

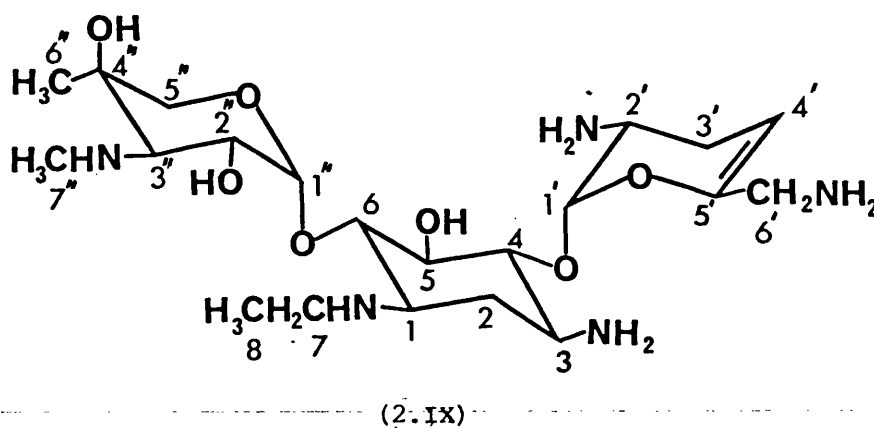


The Chinese sample also produced large amounts of a co-eluting component of gentamicin C₂. This was recognised by unusual Carbon-13 NMR spectra though TLC or paper chromatography could not separate the components. The component, assumed to be gentamicin C_{2a}, was separated analytically by HPLC and estimated to be in equal proportions to gentamicin C₂ (3.3.1). Other components were eluted after gentamicin C_{1a} in large quantities but were likely to be two ring fragments⁷⁷. A field desorption spectrum did reveal components of m/z 450 and 436 which were possibly the same as the demethyl derivatives isolated by Bérdy⁷⁴.

It is interesting to conclude that the different sources of gentamicin sulphate reveal varying minor component profiles, due probably to different conditions of incubation medium or to different strains of producer organism. These components exhibit a wide range of structural modification of the gentamicin C nucleus, some of which are promising in terms of enhancing the aminoglycosides clinical activity. The Chinese sample seems to have been produced in a culture medium with an unusual biosynthetic pathway as the deoxystreptamine is an unorthodox site for N-methylation. Other naturally-occurring aminoglycosides have been isolated with such N-methylation such as Hygromycin B and Destomycin A but in these compounds the deoxystreptamine group is not central and only has one glycosidic bond attached⁴⁶.

2.3.4 Spectroscopic Study of Netilmicin

The observation of two peaks for netilmicin in the HPLC system (3.3.1) which rendered it unsuitable as a standard was probably due to a column or reaction effect. However, to rule out the possibility of a heterogeneous compound an investigation of its composition was made. The structure of netilmicin is given as (2.IX)²⁰.



A chemical ionization spectrum is shown in Figure 2.21, with the $[M+1]^+$ of m/z 476 agreeing with the molecular formula of $C_{21}H_{41}N_5O_7$. Garosamine is present (m/z 160) as is the 4',5'-dehydropurpurosamine C at m/z 127. The N-ethyl deoxystreptamine is indicated by the signal at m/z 191 and the peak at m/z 350 which is the N-ethyl deoxystreptamine attached to garosamine. There is no evidence from this spectrum of an impurity or that the sample is a mixture. The mercaptolysis of N-acetylated netilmicin⁴⁹, though it provided a $[M+1]^+$ peak of m/z 335 attributable to the 2,6-diacetamido-5-keto-hexanal, did not exhibit the expected fragmentation pattern. Hydrolysis and N-acetylation of netilmicin revealed the N,N-diacetyl, N-ethyldeoxystreptamine by a chemical ionization

Figure 2.21. C.I. Mass Spectrum of Netilmicin.

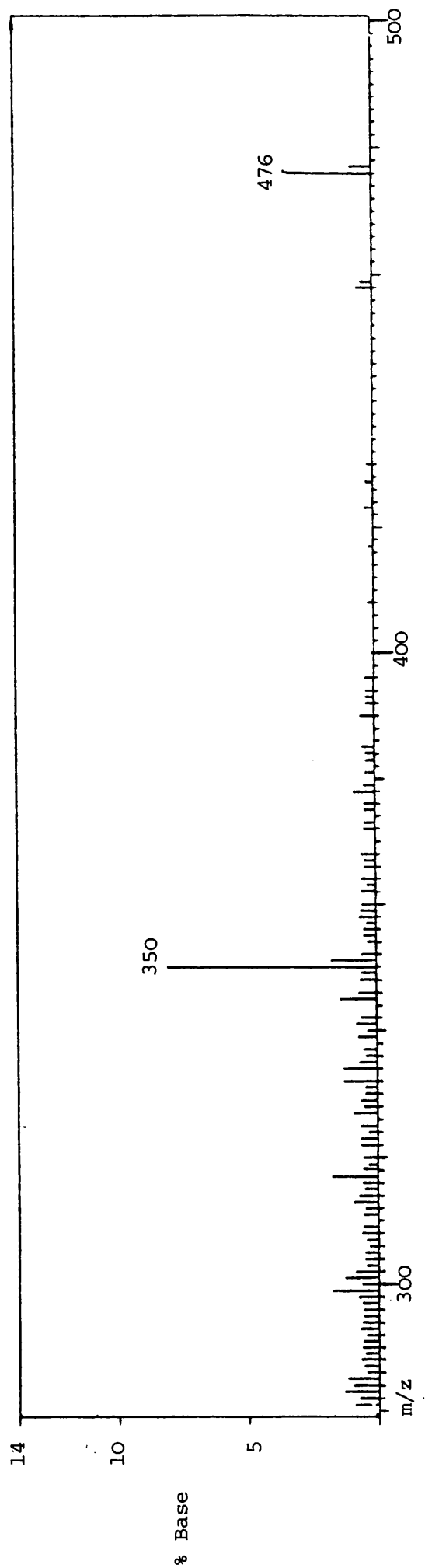
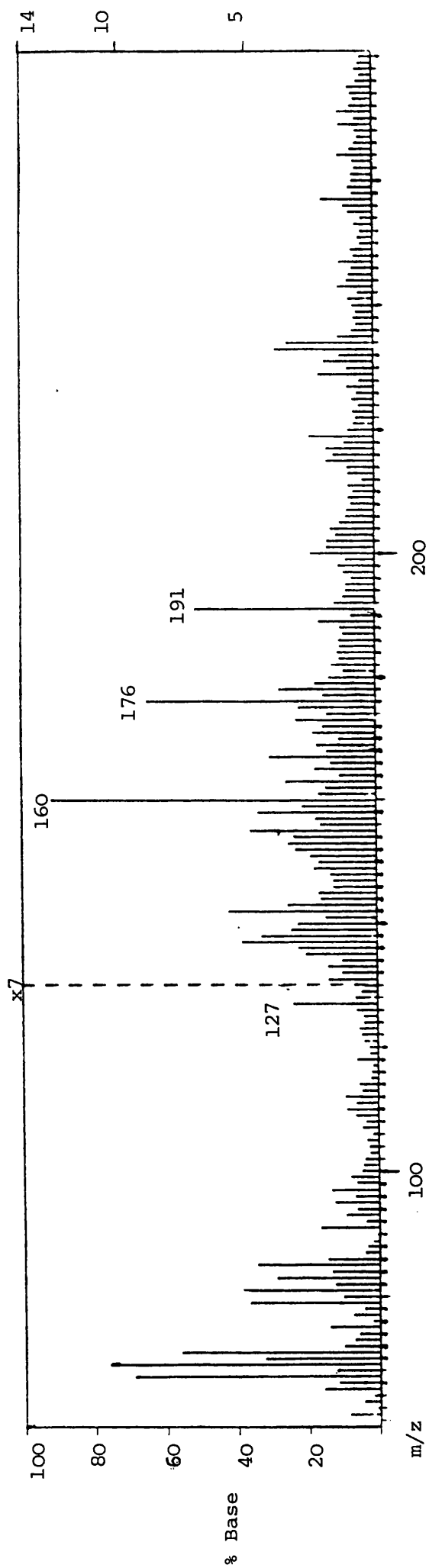
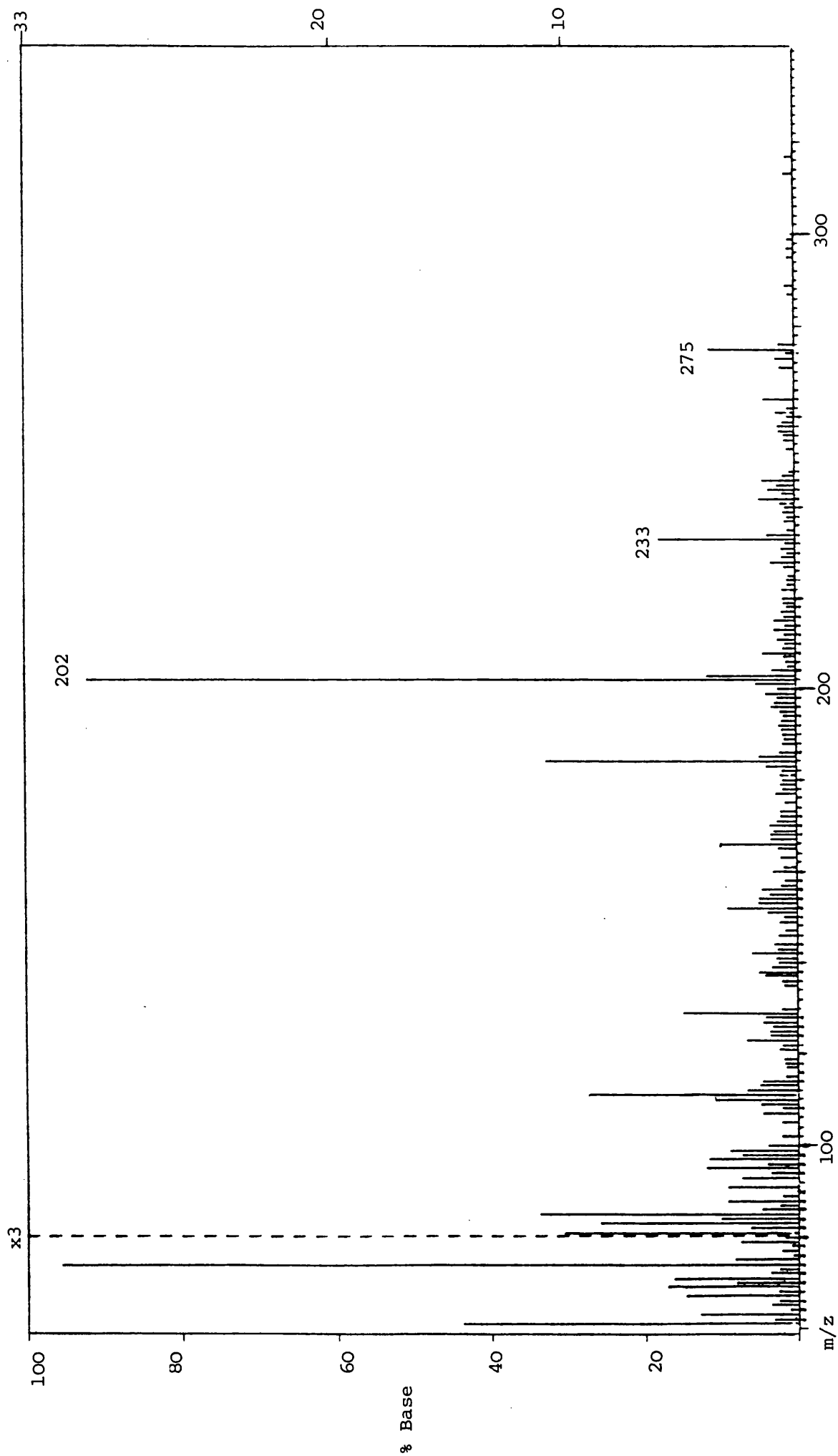


Figure 2.22. C.I. Mass Spectrum of N,N,-Diacetyl-N-Ethyl Deoxystreptamine



spectrum (Figure 2.22). The m/z 275 signal corresponds to the $[M+1]^+$ of this fragment and the large m/z 202 peak is due to an impurity of N-acetylgarosamine.

The carbon-13 NMR spectrum of netilmicin is illustrated in Figure 2.23 with chemical shifts assigned in Table 2.6, and compared to gentamicin C_{1a} . The introduction of unsaturation to the purpurosamine C ring deshields the C-4' and 5' by over 70 ppm which is as expected for such a function. The values for the A-ring are very similar to those reported for sisomicin²⁴⁶. The N-ethyl group appears to influence both the B and C rings. The N-methyl (C-7'') is shielded by 6 ppm although a reason for this is unknown. It is unlikely to be the influence of the unsaturation as sisomicin has been reported to possess chemical shifts of the C ring the same as the gentamicins²⁴⁶. It is most likely caused by a steric factor which may also account for netilmicin's unusual resistance against bacterial enzymes.

The 100 MHz proton spectrum of netilmicin is shown in Figure 2.24. The tertiary methyl (6'') exhibits a signal at δ 1.20 and the N-methyl (7'') at δ 2.52 which are similar to resonances found in the gentamicins. The methylene group in the A ring (6') gives a singlet at δ 3.16 whilst the 4' proton doublet is not distinguishable. The ethyl protons show as a triplet (C-8) ($J = 7$ Hz) at δ 1.06 and a quartet at about δ 2.7 (C-7).

Figure 2.23. Carbon-13 NMR Spectrum of Netilmicin

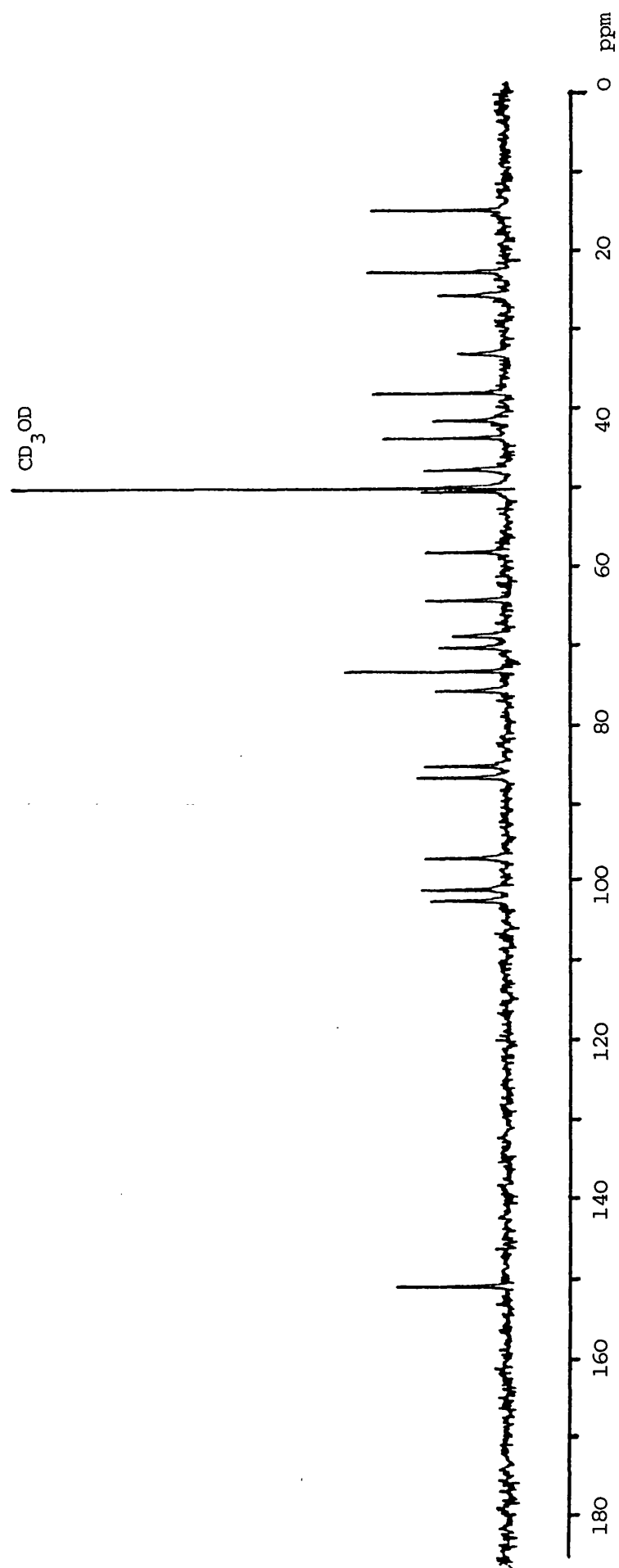


Table 2.6. Carbon-13 NMR shifts of netilmicin in comparison
to Gentamicin C_{1a}.

Carbon	Netilmicin (ppm)	Gentamicin C _{1a} (ppm)
1	57.84	51.95
2	37.53	37.06
3	50.04	50.82
4	86.39	88.14
5	75.40	75.74
6	84.98	88.14
7	22.36	-
8	14.51	
1'	101.94	102.23
2'	47.23	51.14
3'	25.29	27.25
4'	96.58	25.25
5'	149.99	71.32
6'	43.16	46.19
1"	100.53	101.69
2"	71.98	70.59
3"	64.02	64.68
4"	72.96	73.62
5"	69.98	69.00
6"	20.84	23.08
7"	32.55	38.25

Figure 2.24. 100 MHz Proton NMR Spectrum of Netilmicin

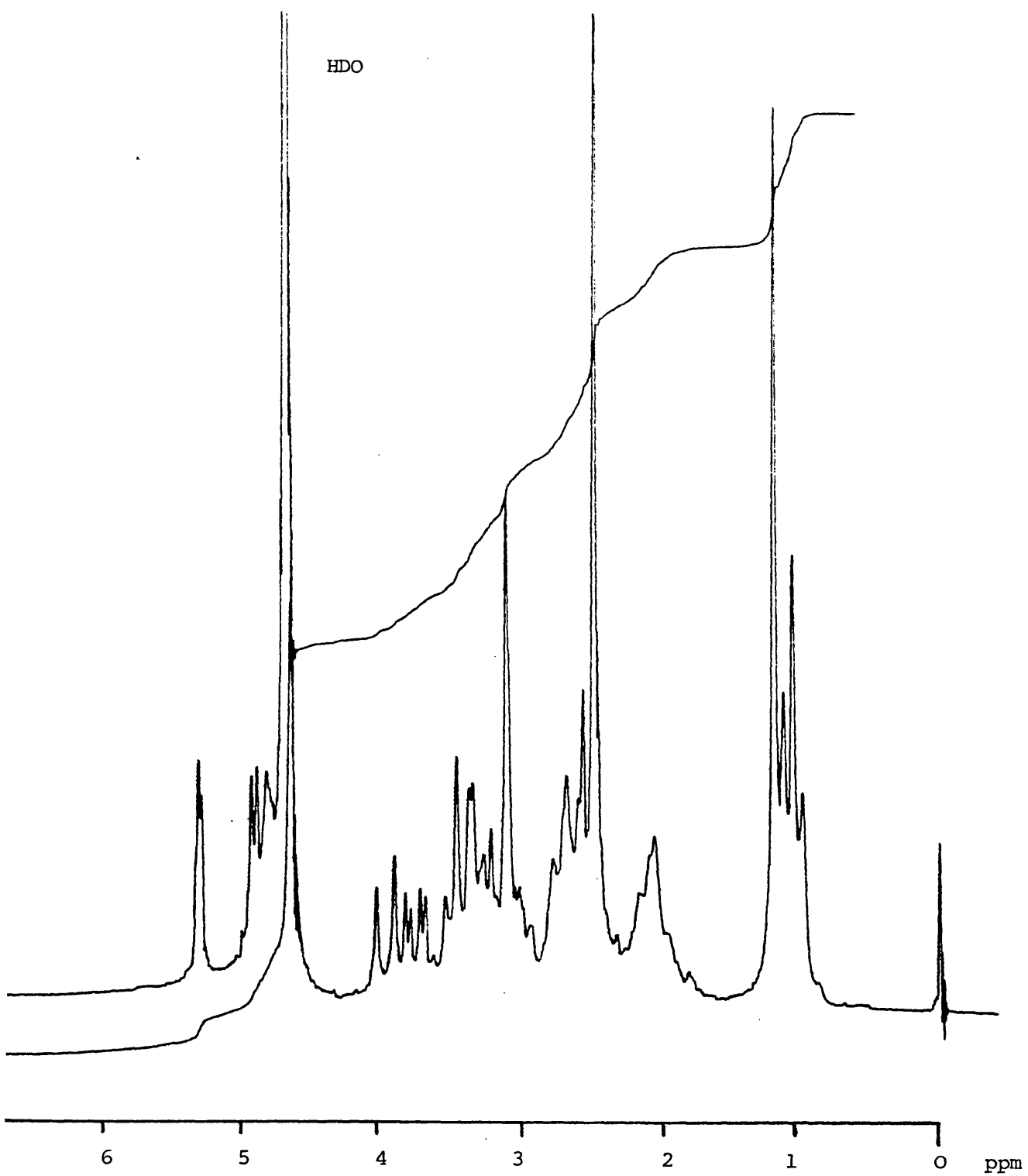
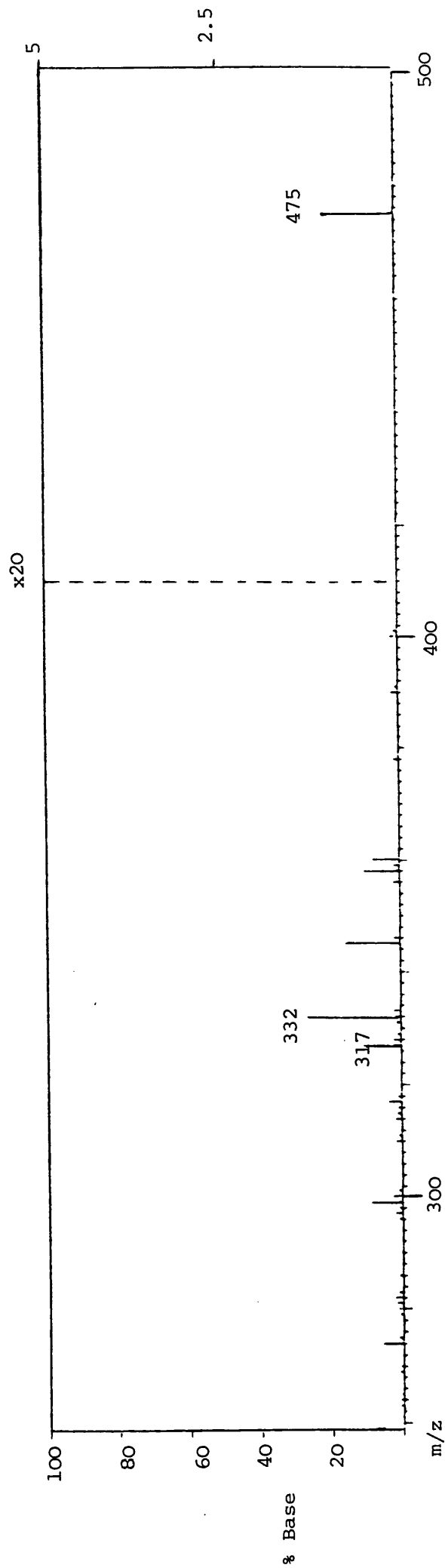
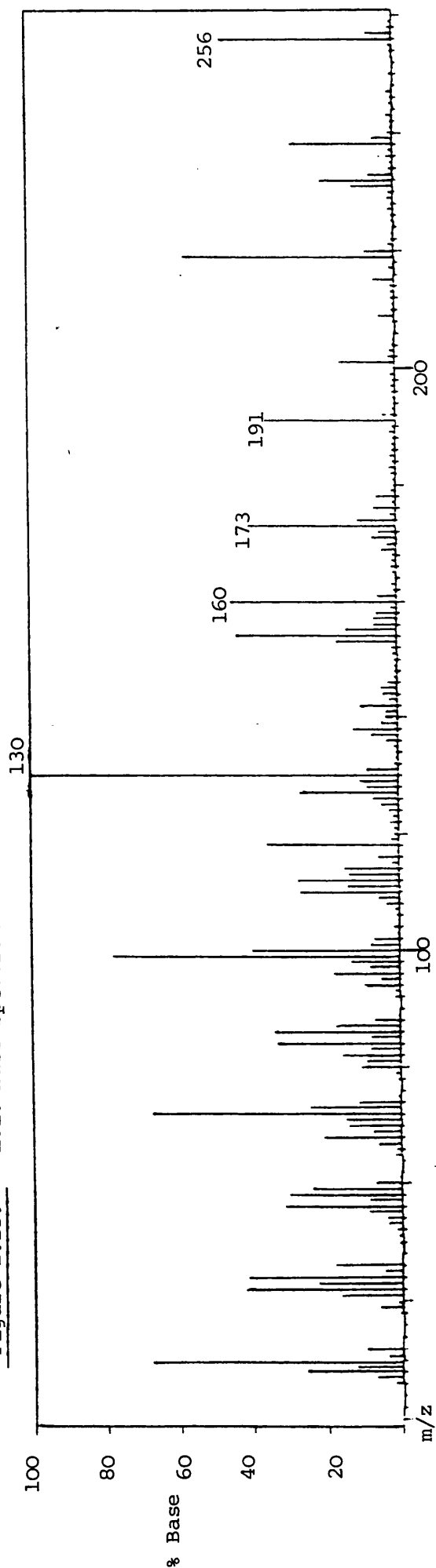


Figure 2.25. E.I. Mass Spectrum of Netilmicin



Both the carbon-13 and proton NMR spectra show no sign of heterogeneity. Applying the mass spectral test of position of N-alkylation²⁰ used in (2.3.3) for component CM5 would yield ions of m/z 317/256 for 1-N-ethylation or m/z 289/284 for 3-N-ethylation. The EI spectrum, Figure 2.25, has peaks at m/z 317 and 256 with no peaks visible at the other positions. It thus demonstrates that the sample was indeed 1-N-ethylsisomicin and no trace of contaminant was detected.

CHAPTER 3

QUANTITATION OF COMPONENT LEVELS OF GENTAMICIN IN COMMERCIAL SAMPLES OF GENTAMICIN SULPHATE AND IN BLOOD PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.1 Introduction

High-performance liquid chromatography (HPLC) has been used in various studies to determine the component ratio of gentamicin sulphate in commercial samples from a wide range of sources^{78,79,82,89}. The advantages of this technique are the speed and precision over more established chromatographic methods such as paper chromatography³¹, thin-layer chromatography⁸⁴⁻⁸⁸ and ion-exchange^{80,83}.

Gentamicin does not possess a chromophore and has to be derivatised before detection is possible. Fortunately the amino groups present offer a wide choice of suitable derivatisation reactions²³⁶. The HPLC methods developed for component determination in commercial samples use ortho-phthalaldehyde (OPA) as the derivatising agent, which yields a fluorescent product^{237,238}. OPA can be reacted with the gentamicin either before or after passing the sample through the chromatographic column²³⁶. The advantages cited for post-column reaction^{239,240} are that formation of artefacts is less likely and product stability is not important. The pre-column reaction enables greater optimisation of the conditions thus allowing a wider choice of reagents and the formed products can have superior separation. Methods which utilise pre-^{78,82} and post-column⁸⁹ reactions with OPA for gentamicin have been reported and both types show good precision.

These chromatographic techniques generally separate the samples into the three major components, gentamicin C_1 , C_{1a} and C_2 . However, Freeman⁸² developed an OPA pre-column derivatisation technique which could also separate the gentamicin C_{2a} component. This was achieved by altering the composition of the fluorescent reagent and adding an ion-pair compound to the mobile phase. White et al.⁷⁹ used this method to investigate the relative component composition of gentamicin sulphate from various geographical sources. The availability of the HPLC method enables more stringent analysis of samples of different origin and thus the possibility of a greater standardisation of the component composition especially in respect to gentamicin C_{2a} .

The development of methods of plasma analysis of gentamicin by HPLC has been reported more frequently²²⁰⁻²³⁵, perhaps because of the more exacting conditions imposed, or because of a more apparent need. More reagents have been used to derivatise the amino groups than just OPA, and ultra-violet detection used as well as fluorescence^{228,229}. Reagents used for pre-column derivatisation include fluorescamine²³⁵, dansyl chloride²²⁰⁻², benzene sulphonyl chloride²²⁹ and fluorodinitrobenzene²²⁸. For some of these reactions the individual component derivatives were not totally separated by the column^{220,222,228,229} and this could lead to inaccurate results if a mixture of different composition from that used in the calibrations was assayed. Only OPA has been used for post-column derivatisation of gentamicin²²⁵⁻⁷ because of the severe constraints imposed

on post-column reactions.

Separation of the gentamicin from the plasma constituents is an important procedure, as gentamicin is slightly bound to protein and biogenic amines can readily react with the reagent and cause interference. Two types of separation can be used; either adsorption or extraction. The former has been achieved for gentamicin by use of silicic acid or ion-exchange resins before reaction with the reagent^{223-226,233}. The other type of separation used was protein precipitation followed by solvent extraction with a variety of methods developed^{220-2,227-232}. Many published methods rely on external standardisation because of the difficulty in obtaining suitable internal standards. Tobramycin and netilmicin have also been assayed by HPLC using similar techniques^{221,224,230-32} and in some cases can be used as internal standards in gentamicin assays^{229,233}.

All developed methods claim good precision with values of less than 10% coefficient of variation¹⁶². Another advantage of HPLC is the specificity, since no compounds have been reported to overlap with the gentamicin components¹⁶². The quantity of plasma required for a single analysis varied from 0.02 to 0.5 ml in the reported assays and thus allows sampling from infants. The time required for a single assay depends on the method, but was in a range of about fifteen to seventy minutes. The absolute time limitation is the retention time on the column. The sensitivity of the methods extended to at least 0.5 µg/ml which is adequate for clinical use.

The aim of the work in this chapter was to develop a pre-column OPA derivatisation HPLC method for gentamicin component analysis and apply it to commercial samples from differing sources. Also some of the reported methods of HPLC determination of gentamicin in plasma were investigated. Using a developed plasma method, a comparison with the microbiological assay was made and pharmacokinetic parameters of the three components calculated.

3.2 Materials and Methods

3.2.1 Materials

Ortho-phthalaldehyde (Sepramar), boric acid, potassium hydroxide, mercaptoethanol, trichloroacetic acid and anthracene were all supplied by BDH Chemicals Ltd. Ethylenediaminetetraacetic acid (tripotassium salt), tetradecylamine, tridecylamine and undecylamine were supplied by Fluorochem Ltd.. Nonylamine was obtained from Aldrich Chemical Company Ltd., Dodecylamine from Eastman Kodak Company and Decylamine from Sigma Chemical Company. HPLC grade methanol and diethyl ether were obtained from Fisons Scientific Apparatus Ltd. Filters of 0.45 μ m and prefilters were obtained from Millipore (UK) Ltd. Netilmicin sulphate was a gift from Kirby-Warrick Pharmaceuticals Ltd. Gentamicin components (C_1 , C_{1a} and C_2) had been isolated by column chromatography and purity was checked by TLC (2.2.2). The pump used was a Laboratory Data Control Minipump and the detector was a LDC Fluoromonitor III (excitation filter 370 nm, emission filter 418 - 700 nm). The injection valve used was

a Rheodyne 7125 with a 100 μ l loop and the recorder was a Servoscribe RE 541.20. A Hewitt and Packard 3903 integrator was also used in some component assays. The column used contained Spherisorb ODS, 5 μ m, and had dimensions of 150 x 5 mm.

3.2.2 Methods

3.2.2a General HPLC Method

Chromatographic Conditions:

The mobile phase used was methanol/water (80:20) with 2 g/L EDTA, tripotassium salt. The flow rate was 1.3 ml min⁻¹ and the column temperature was maintained at 30°C.

Derivatising (OPA) Reagent²²³:

Ortho-phthalaldehyde (OPA) (200 mg) and mercapto-ethanol (0.4 ml) were dissolved in methanol (2 ml). Boric acid (1 g) was dissolved in distilled water (38 ml) and the pH was adjusted to 10.4 with 45% w/v potassium hydroxide solution. The entire buffer solution thus prepared was then added to the OPA methanolic solution. The reagent was stored under nitrogen at 4°C and was stable for at least two weeks.

Reaction Conditions:

The basic reaction consisted of adding OPA reagent (0.5 ml) to an aqueous solution of gentamicin (0.01 mg/ml) (0.5 ml)

and after vortex mixing for one minute, methanol (2 ml) was added. The solution was filtered and stored at room temperature, in the dark until injection.

The reaction conditions were varied to optimise yield. The length of time of reaction was varied up to 120 minutes for each of the individual components. The temperature was altered up to 50°C and the effect of lowering the pH of the reaction mixture was also investigated.

Internal Standards

Arginine, anthracene, nonylamine, decylamine, undecylamine, dodecylamine, tridecylamine, tetradecylamine and netilmicin were all examined as possible internal standards. Kappa' (κ') values were calculated for each from

$$\kappa' = \frac{t_r - t_o}{t_o}$$

where t_o = leading edge of first deflection (unretained)

t_r = centre of peak (retained)

3.2.2.b Gentamicin component ratio determinations in commercial material samples and pharmaceutical preparations

Gentamicin Standard Solutions (Raw Material Analysis)

Each of the three gentamicin C components was dissolved

in water to produce 2 mg/ml standard solutions. These were further diluted with water to give a range of solutions of 20, 30, 40, 50, 60, 70 and 80 µg/ml for each of the components.

Standard Solution for Injection Analysis:

From the 2 mg/ml standard solutions of the components, 0.27 ml of gentamicin C_{1a}, 0.33 ml of gentamicin C₂ and 0.40 ml of gentamicin C₁ were taken and diluted to 5 ml to give a 0.4 mg/ml solution containing all the components though not of equal concentration.

Internal Standard Solutions:

Anthracene 0.06 mg/ml in methanol, undecylamine and tridecylamine 0.1 mg/ml in methanol, solutions were prepared.

Preparation of Commercial Material Samples:

Gentamicin sulphate (25 mg) was dissolved in water (25 ml). A 1 in 10 dilution of this solution was made and used in the assay. Duplicate solutions of each sample were prepared.

Preparation of Injectable Formulation

Gentamicin Injection (80 mg in 2 ml) (0.1 ml) was diluted to 10 ml with water to give a 0.4 mg/ml solution.

Duplicates from the same ampoule were prepared.

Calibration - Gentamicin Commercial Material

Calibration solutions were prepared by adding 0.3 ml gentamicin C_{1a} standard solution to 0.36 ml of gentamicin C₂ solution and 0.44 ml of gentamicin C₁ solution, all of the same strength (10 - 80 µg/ml). Undecylamine (0.1 ml) and tridecylamine (0.1 ml) were added followed by OPA reagent (0.5 ml) and methanol (2 ml). The solution was vortex mixed, filtered and stored in the dark and injected (100 µl) 60 minutes after the reaction had started. This was the standard procedure for the reaction, and kept to unless otherwise stated.

Gentamicin Sulphate Injection: Calibration

Solutions were prepared by adding varying volumes (60 - 140 µl) of the gentamicin, 0.4 mg/ml standard solution, to water to produce 0.2 ml. Then undecylamine (0.1 ml), OPA reagent (0.5 ml) and methanol (2 ml) were added, and the reaction carried out as above. In most cases a five point calibration was used.

Gentamicin Commercial Material Assay

Samples were analysed by the standard method using unknown 0.1 mg/ml solution (1.1 ml), undecylamine (0.1 ml), tridecylamine (0.1 ml), OPA reagent (0.5 ml) and methanol (2 ml).

Gentamicin Injection Assay

Samples were assayed by adding the approximately 0.4 mg/ml solution of the unknown (0.1 ml) to water (0.1 ml) and undecylamine (0.1 ml). To this OPA reagent (0.5 ml) and methanol (2 ml) were added and the usual procedure followed.

3.2.2.c Assay of Gentamicin Levels in Blood Plasma

Plasma

Blood plasma used in the development of the method and for assay calibration was obtained from the Royal United Hospital, Bath. It was from a pool of healthy subjects with no drugs present. Plasma was spiked with gentamicin standard solution, 0.4 mg/ml, the day before use and stored frozen at -20°C .

Gentamicin Extraction from Plasma onto a Silica Gel

Column²²³

A short silica gel column was prepared by blocking a pasteur pipette with glass wool and filling the pipette with 150 mg of silica gel (100 - 200 μm). Water was added to plasma (0.5 ml) to give a 2 ml volume. This solution was transferred to the column and washed in with 1 ml of water. OPA reagent (0.5 ml) was added to the column and the OPA derivative of gentamicin was eluted with 1.5 ml of ethanol after ten minutes. This solution was filtered and injected after fifteen minutes.

Protein Precipitation with Acetonitrile; Extraction
with Ethyl Acetate²³¹

Plasma, spiked with gentamicin (0.2 ml) was added to 0.1 M phosphate buffer (pH 11.0) (0.8 ml). To this acetonitrile (3 ml) was added, vortex mixed, and centrifuged for 10 minutes at 3,000 rpm. All of the supernatant was then added to methylene chloride (3 ml), mixed gently, then centrifuged for 5 minutes at 2,000 rpm. Then aqueous phase (0.5 ml) was taken and added to OPA reagent (0.5 ml). This was mixed and left for 5 minutes before ethyl acetate (0.5 ml) was added. The mixture was centrifuged at 1,000 rpm for one minute. Organic layer (100 µl) was then injected immediately after separation. For some samples isopropanol (0.25 ml) and sodium carbonate (500 mg) were used at the final extraction step instead of ethyl acetate.

Protein Precipitation with Trichloroacetic Acid;
Extraction with Diethyl Ether

Trichloroacetic acid (10% w/v in 80:20, methanol: water), 0.25 ml, was added to plasma (0.5 ml). This was vortex mixed for one minute then centrifuged for ten minutes at 3,000 rpm. Total supernatant was added to 0.4 M borate buffer (pH 10.4), (0.25 ml) and OPA reagent (1 ml). This was mixed and left for twenty minutes in the dark. To the solution diethyl ether (5 ml) was added, lightly mixed, then centrifuged at 2,000 rpm for 5 minutes. The upper etherial layer was separated, evaporated under nitrogen and the residue redissolved in 1:1 methanol:water (0.5 ml). This solution (100 µl) was injected immediately.

Protein Precipitation with Trichloroacetic Acid -

No Extraction

Trichloroacetic acid (10% w/v in 80:20;methanol:water), (0.25 ml), was added to plasma (0.5 ml). This was vortex mixed for one minute, then centrifuged for ten minutes at 3,000 rpm. Total supernatant was added to 0.4 M borate buffer (pH 10.4), 0.25 ml, and OPA reagent (0.5 ml). Methanol (0.8 ml) was added and the solution was prefiltered, filtered and injected (100 μ l) after sixty minutes.

Plasma Assay Calibration

Blood plasma (2 ml) was spiked with gentamicin standard solution, 0.4 mg/ml, to give 1, 2.5, 5, 7.5 and 10 μ g/ml solutions of gentamicin in plasma. These were processed, in duplicate, by the method of trichloroacetic acid precipitation with no extraction except that 4 μ l undecylamine (0.1 mg/ml) as internal standard was added after protein precipitation.

Assay of Plasma Samples

The patient samples were prepared in an identical fashion to the calibration solutions. To avoid the need for daily calibrations, standard solutions were assayed after every two unknown sample injections. About nine samples, each in duplicate, were analysed every day. In total sixty five samples from five patients were used and assayed at random. At the end of each day the HPLC column was eluted with methanol to prevent

build-up of contaminants.

3.3 Results and Discussion

3.3.1 Determination of Component Levels in Commercial Material

Optimising Conditions:

The HPLC system used, was based on that reported by Maitra *et al.*²²³ with some modifications. The mobile phase was a 80:20, methanol-water mixture containing 2 g/L ethylene-diaminetetra-acetic acid, tripotassium salt. This gave a typical separation such as that illustrated in Figure 3.1. The retention times of the components were 5, 6 and 12 minutes for gentamicins C_{1a}, C₂ and C₁ respectively. No buffer was used as the salt was found to produce a fairly constant pH of 8.5 in the mobile phase. Extensive variation of the mobile phase is discussed in Chapter 5.

The reaction conditions of the derivatising reagent were studied to optimise the sensitivity. The peak height of the three components was measured against time of incubation at room temperature with a graph of the results shown in Figure 3.2. The components showed different reaction kinetics with the rate possibly influenced by stereochemical factors. Sixty minutes was taken as the optimal time since two components had reduced peak height after this juncture. The use of elevated temperature (50°C) was examined, but the rate of decomposition of the product was also increased and timing was more critical. The reaction was thus kept at room temperature to minimise

Figure 3.1. HPLC Chromatogram of Gentamicin Components
(Spherisorb Column)

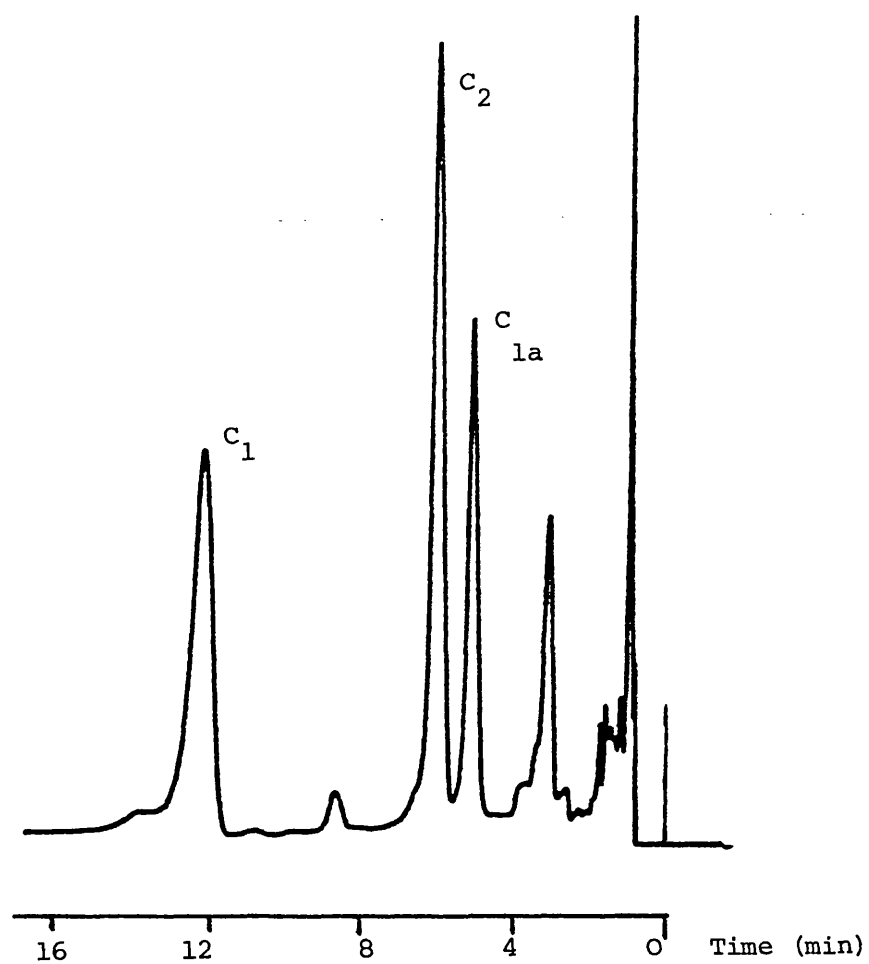
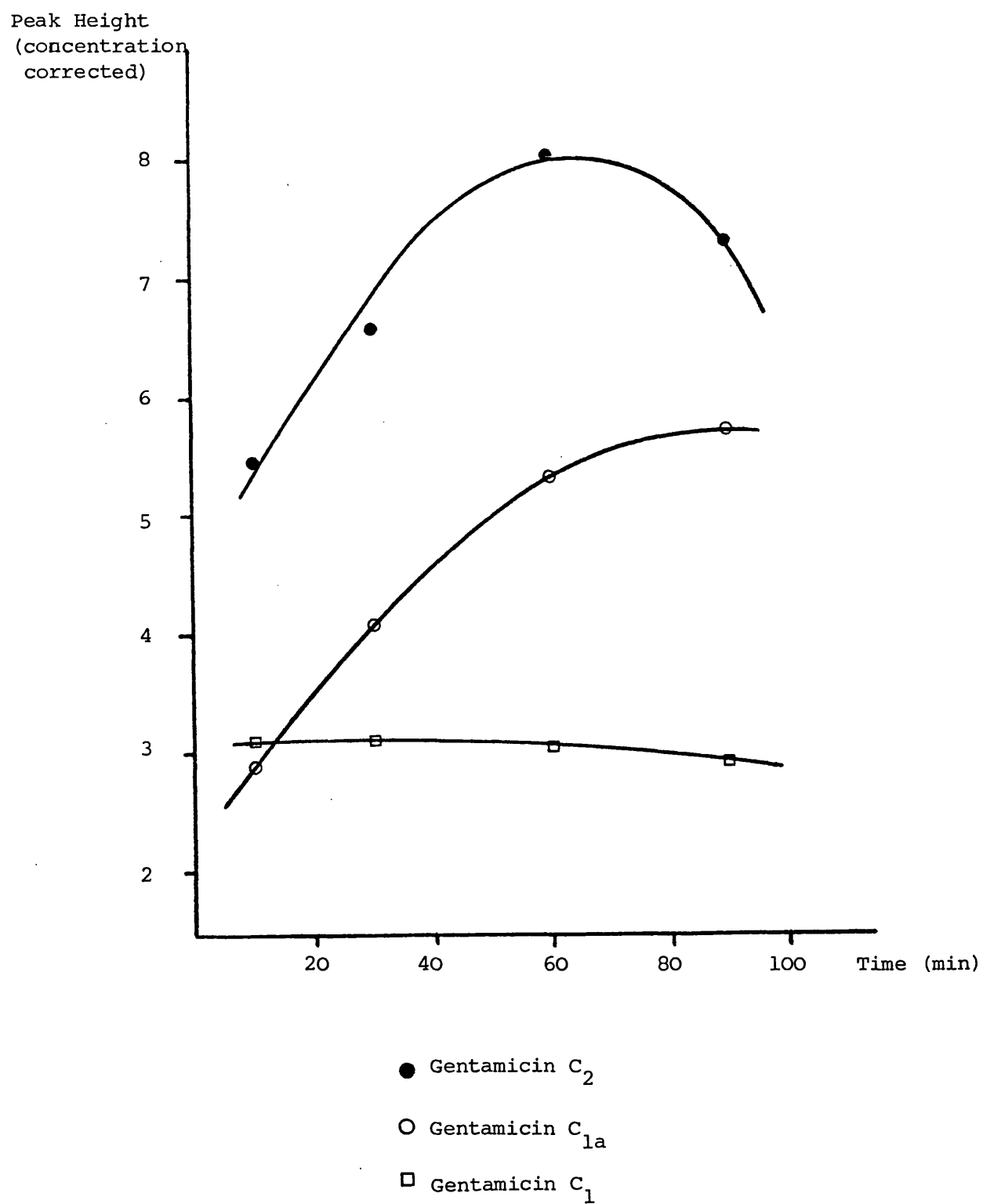


Figure 3.2. Time Course of Gentamicin-OPA Reaction with
respect to Peak Height



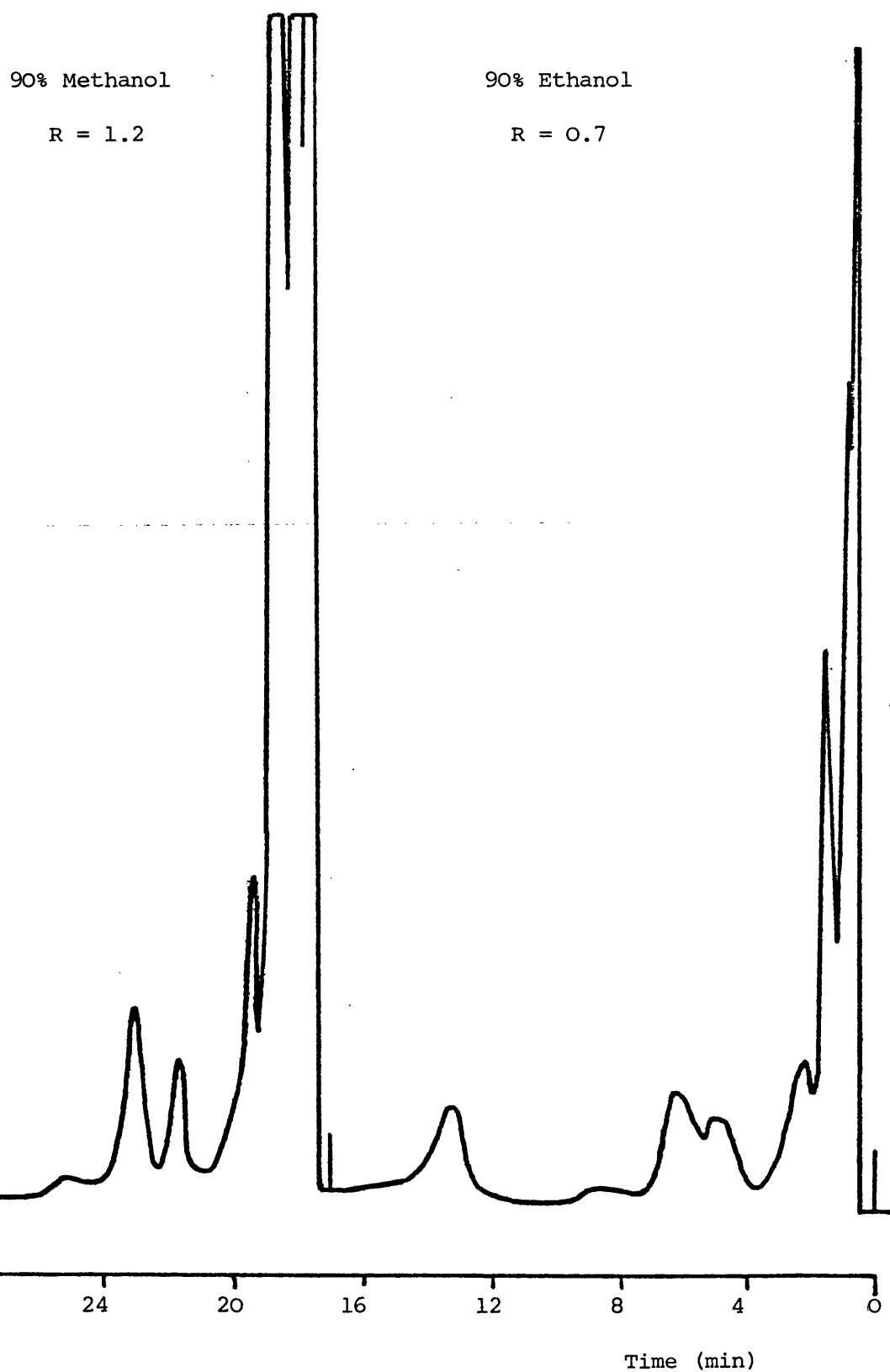
variation in reaction yields.

The addition of an alcohol to the aqueous reaction mixture increased the fluorescence produced. The resolution of the gentamicin C_{1a} and C_2 peaks in a chromatogram was 1.2 in a 90% methanol solution as shown in Figure 3.3. This compares to a resolution of 1.0 in a 90% (methanol/ethanol (1:1)) mixture and 0.7 for 90% ethanol or isopropanol reaction solutions. For methanolic solutions there is less than a 1% overlap of peaks, if taken as gaussian in shape, which is satisfactory for quantitative determinations²⁴¹. The poor resolution in the other alcoholic reaction solutions was probably related to the increase in solvent strength at the head of the column, on injection, eluting the derivatives slightly faster and with poorer separation²⁴¹.

The pH of the reaction mixture was not found to be critical so long as conditions remained alkaline and thus the OPA reagent used was as described by Maitra et al.²²³, with a pH of 10.4. A pH range of 8 - 12 for the reaction gave peak heights of similar magnitude but incubation at an acid pH (0.6) only produced 13% peak size. Alteration of the pH thirty minutes after reaction was started also showed the derivatives to be stable if the pH was kept alkaline. Changing the pH to 3.9 decreased peak heights by 14%; and to 2.0 by 49% after thirty minutes in comparison to the control.

To minimise variation in experimental procedure a set time

Figure 3.3. Resolution of C_{1a} and C_2 components injected
in different reaction mixtures



of sixty minutes at room temperature, with the same pH 10.4 buffer and the same percentage volume of methanol were used in the reaction.

Internal Standards:

The standard used by Kraisintu^{77,78}, L-arginine was tested, but found to elute with a retention time of about two minutes and overlapped peaks due to the derivatising reagent. Various aliphatic and aromatic amines and naturally fluorescing compounds were investigated but most were non-retained. Netilmicin was also examined but under these conditions it eluted as two peaks. This was possibly due to a partial reaction as the relative size of the peaks depended on the age of the reagent. Other aminoglycosides tried, including neomycin and kanamycin, were not retained sufficiently in this system to be of use as standards.

Anthracene was discovered to have an ideal retention time and have good peak shape in the system used. The chromatogram of anthracene with the gentamicin components is shown in Figure 3.4. However as a standard this only monitors variations due to volume of injection, Since it does not react with the derivatising reagent it does not control variations due to quality of reagent or reaction conditions. Freeman⁸² used nonylamine in an assay of gentamicin, but in the conditions used here, this overlapped with the gentamicin components. A range of straight chain amines were chromatographed to assess suitable

Figure 3.4. HPLC chromatogram showing Anthracene with gentamicin components

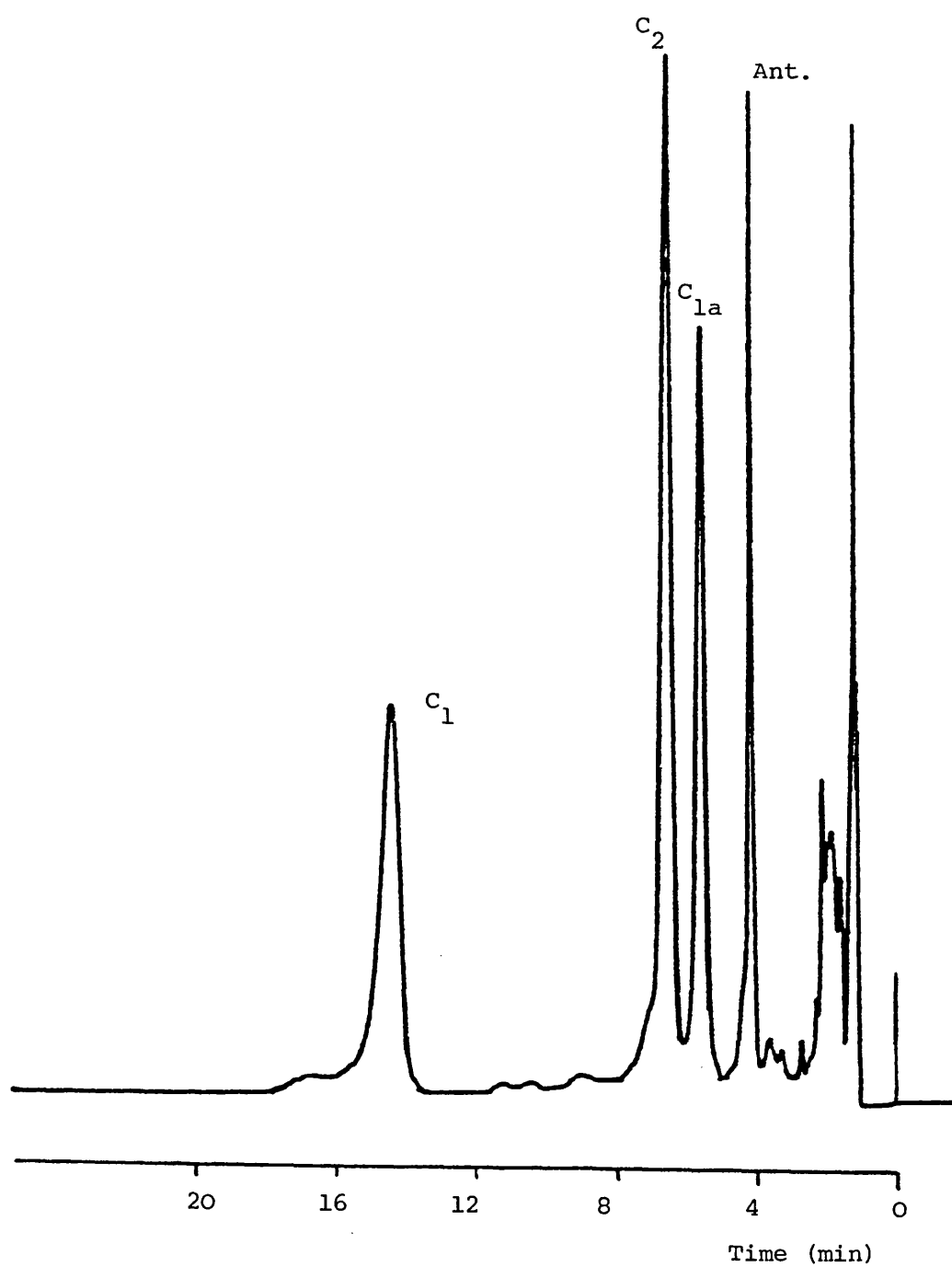
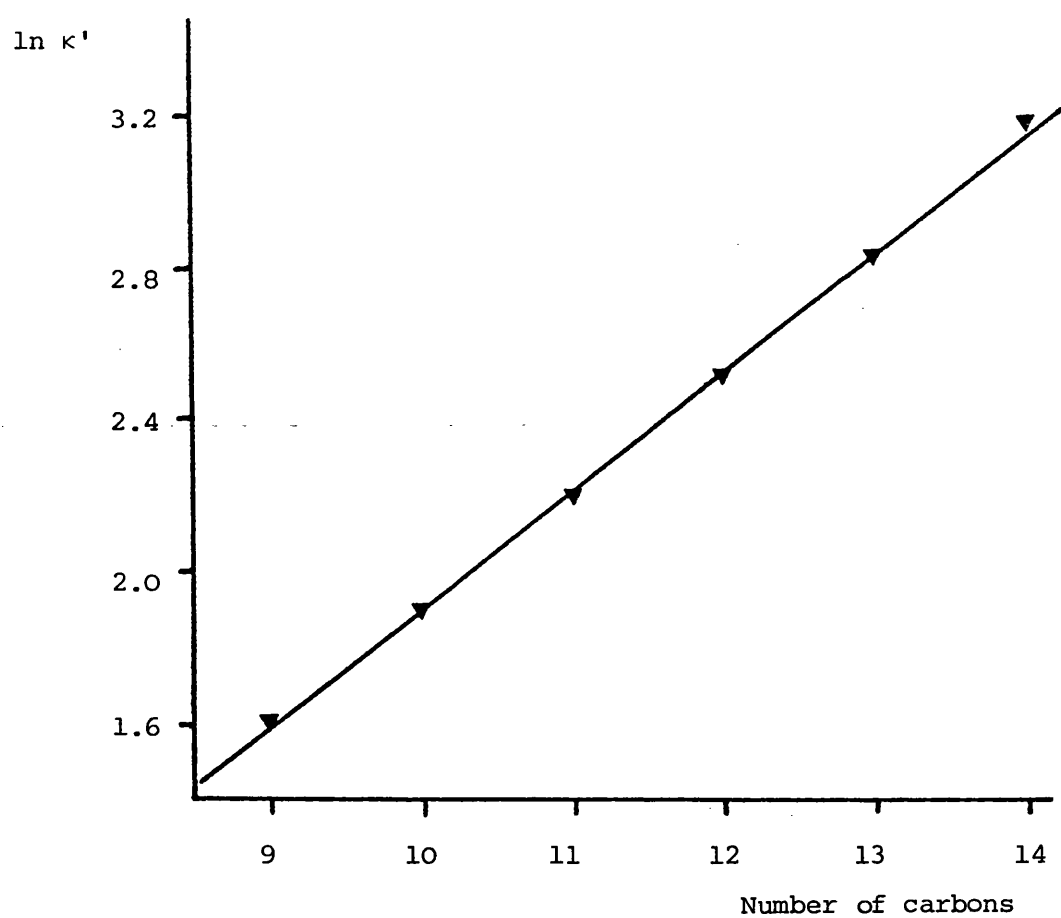


Figure 3.5. Graph of $\ln \kappa'$ against number of carbon atoms for a series of n-alkylamines



retention times. A plot of $\ln k'$ (capacity factor) against number of carbon atoms is shown in Figure 3.5. It is seen to follow the Martin rule for a series of structural homologues^{241,319}. The slope of 0.31 which refers to the increment of added methylene groups is constant for the given system and column. It is similar in value to the estimated slope of 0.40 from a graph presented by Freeman⁸². This illustrates the similarity of the two systems in terms of chromatographic response in spite of all the differences in conditions employed. The amines with retention times different to the gentamicin components were undecylamine, tridecylamine and tetradecylamine. Tetradecylamine was considered unsuitable because of the long retention time (25 minutes) and broader peak shape. The peak area ratio of gentamicin C component to that of undecylamine, against time of reaction is shown in Figure 3.6. The plot of peak height for the same relationship resembles that for peak area. The onset of an approximately constant ratio is obtained after sixty minutes. At times less than this the gentamicin peak heights are increasing whilst that of undecylamine is decreasing.

Calibrations and Assay:

Anthracene was initially used alone as an internal standard with the slopes and intercepts of calibrations calculated by regression analysis. A calibration graph obtained using anthracene is illustrated in Figure 3.7, where peak height ratios were used. Peak area calibrations were also

Figure 3.6. Ratio of Gentamicin Component to Undecylamine
(Peak Area) against time

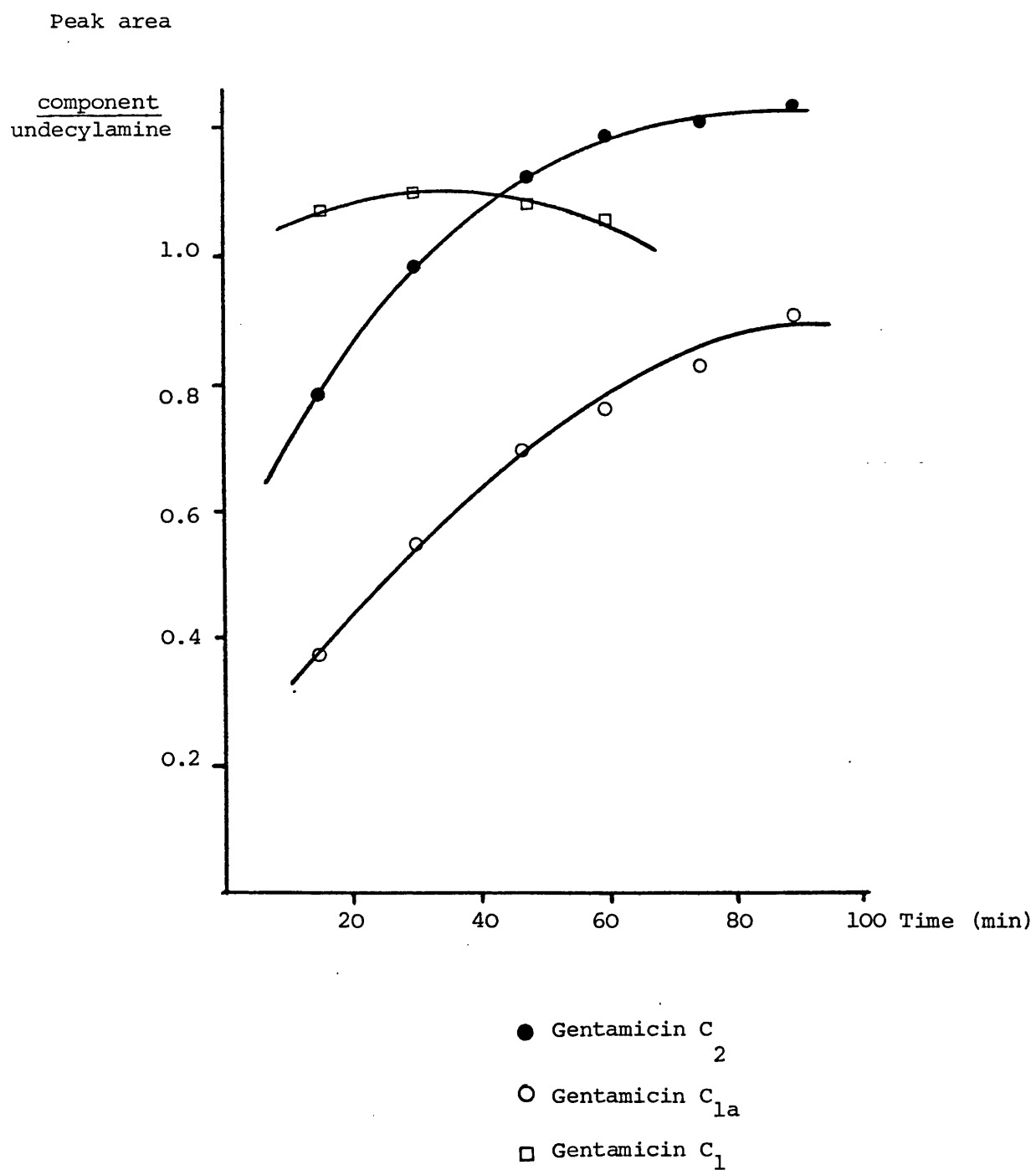
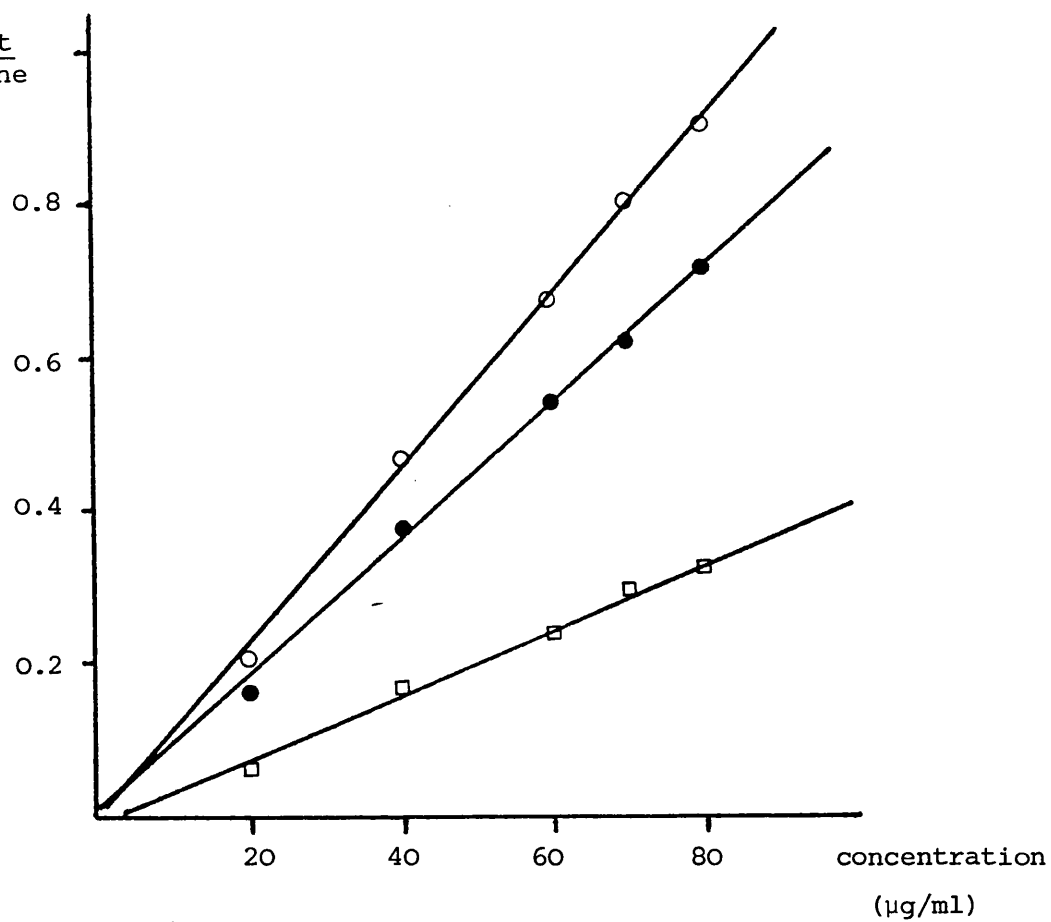


Figure 3.7. Aqueous Calibration of Gentamicin Components
using Anthracene as Internal Standard

Peak Height

$\frac{\text{component}}{\text{anthracene}}$



- Gentamicin C_{1a}
- Gentamicin C₂
- Gentamicin C₁

simultaneously performed to assess the relative precision of the two calibration methods. Four calibrations on consecutive days were compared with the height measurement showing a 7% variation in the slopes determined whilst the variation was 13% for the area measurement. Despite this area determinations were continued with in case of an alteration in component retention. Anthracene, when used in the assay of the gentamicin sulphate commercial material, produced results with large standard deviations. This error was thought to arise because of the absence of reaction of anthracene with the OPA reagent, limiting the compound to a standard of volume but not of the reaction. It was therefore apparent that anthracene was only partially successful as a standard and that an amine which reacted with OPA was required.

Retention data shown in Figure 3.5 suggested that undecylamine or tridecylamine might be suitable to act as an internal standard. Accordingly calibrations were carried out with both these amines present to assess their suitability. A chromatogram of the two standards with the gentamicin components is shown in Figure 3.8, with undecylamine eluting as the third peak and tridecylamine as the last. Results using tridecylamine as standard were subject to large coefficients of variation, but undecylamine showed higher precision, probably because of its narrower peak width. A peak height calibration using undecylamine is shown in Figure 3.9. The mean of four such calibrations was taken for both peak height and area, and used in the assay of component ratios. The figures used are shown in Table 3.1.

Figure 3.8. HPLC Chromatogram of Undecylamine and
Tridecylamine with Gentamicin Components

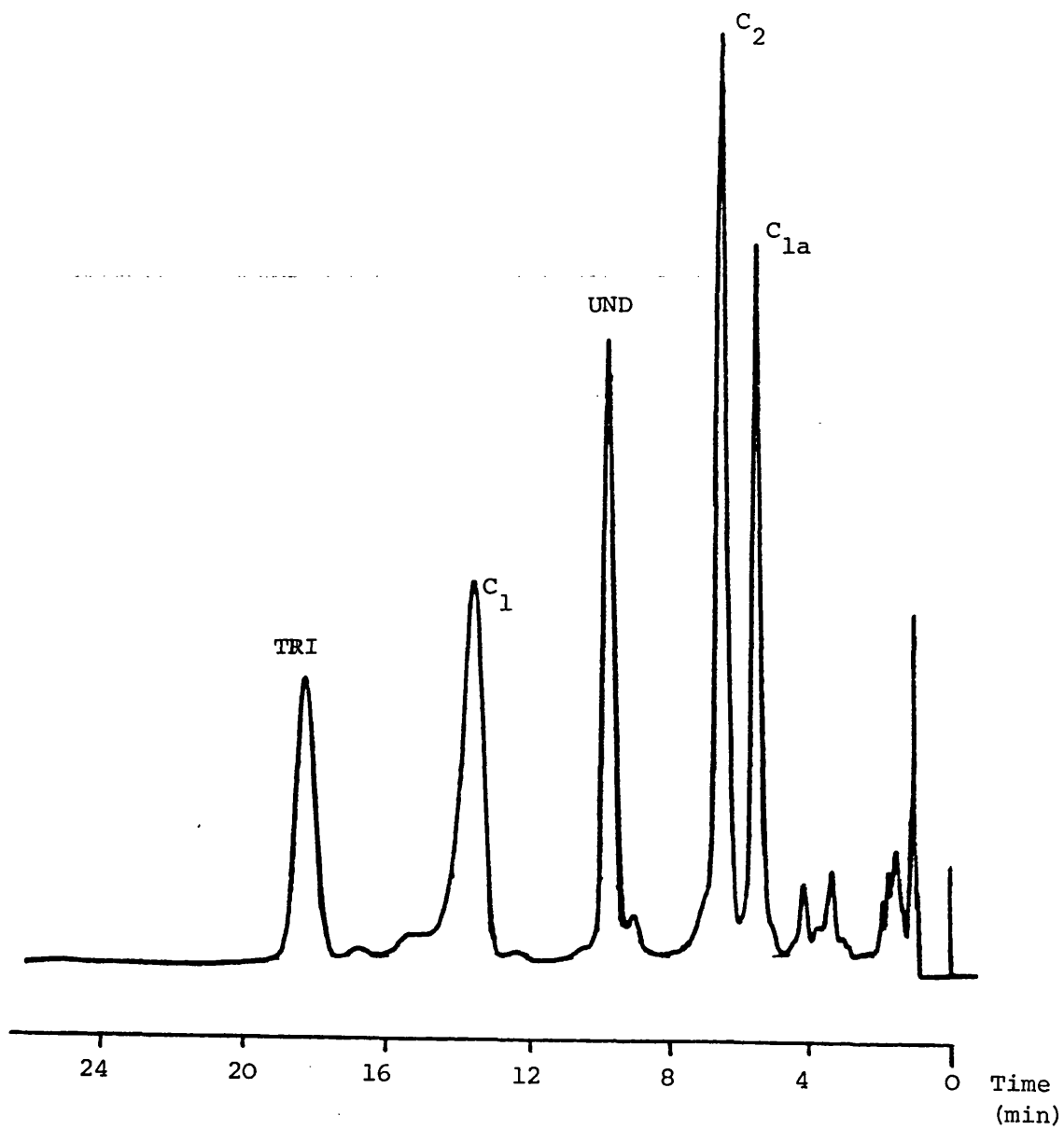
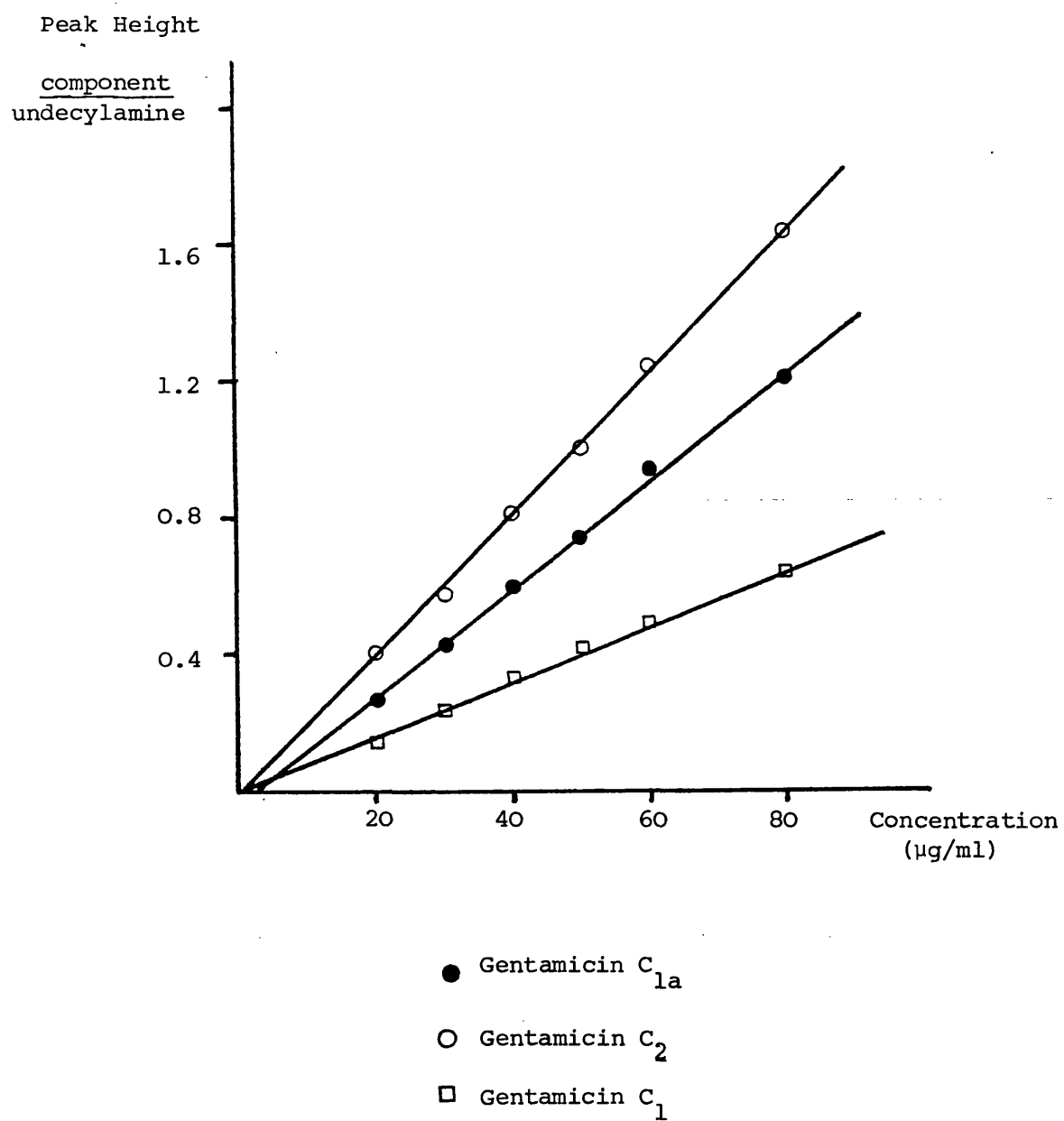


Figure 3.9. Aqueous Calibration of Gentamicin Components
using Undecylamine as Internal Standard



The larger variation in the gentamicin C_1 calibration is probably because of the poorer peak shape in relation to the other two components.

Table 3.1. Undecylamine Calibration Means (n = 4)

	PEAK HEIGHT			PEAK AREA		
	Gent C_{1a}	Gent C_2	Gent C_1	Gent C_{1a}	Gent C_2	Gent C_1
Slope	0.01517	0.02016	0.00754	0.01278	0.01810	0.01485
Intercept	-0.00761	0.00107	0.04624	-0.00801	0.00871	0.0779
% CV	4.6	2.0	15.2	7.2	7.6	12.4

These calibration figures were used to calculate the levels of components by the equation:

$$\text{Concentration of Component} = \frac{\text{Ratio-intercept}}{\text{slope}} \times \frac{a \times F \times 0.88}{1.1} \times \frac{100}{56} \times \frac{25}{\text{wt}}$$

here ratio is determined ratio of component to undecylamine

a is fraction of component present in calibrations

$$(C_{1a} = 0.3, C_2 = 0.36, C_1 = 0.44)$$

F is weight factor of component standard solution

wt is weight of sample in the 25 ml solution

0.88 refers to proportion of moisture in standard

$\frac{100}{56}$ is the conversion of the base to sulphate

The conversion factor of base to sulphate was calculated as follows: The sulphate content of the samples was assumed

to be 32% w/w from reported results^{77,78}. The percentage moisture content was set at 12% as determined by the supplier using the Karl-Fischer titration method³⁰ and confirmed by loss of weight on drying determination³¹. Thus 44% of the sample was thought to not be gentamicin base. The separate gentamicin components used to produce the standard solutions were also found to contain about 12% moisture. This could not be accurately determined because of the low sample weight available (50 - 150 mg) and also the melting of the individual components at 105°C presented experimental difficulties.

The component composition of six samples of gentamicin sulphate powder of two manufacturing sources and one sample of possible Chinese origin were determined. The former six materials were assayed on three separate days in duplicate by peak area and height measurement and all results combined to give the mean value. The Chinese material was only assayed by height measurement, repeated five times. The results are shown in Table 3.2.

Table 3.2. Component Levels in Gentamicin Sulphate Material

Sample	(%) C _{1a}	(%) C ₂	(%) C ₁	(%) Total C	Stand Dev.
A1	26.0	38.9	35.1	100.0	2.4
A2	26.3	40.6	37.3	104.2	3.1
A3	24.3	38.5	35.0	97.8	3.2
B1	27.2	36.2	30.2	93.6	3.8
B2	24.9	40.5	29.3	94.7	4.2
B3	26.9	37.7	28.7	93.3	3.1
Chinese	18.9	37.3	19.7	75.9	2.7

The standard deviation was greater than expected and probably was a combination of the variation in the three separate calibrations required for the calculated total, and the interday variation. The three samples from manufacturer 'A' are similar in composition and show a higher gentamicin C_1 content than the samples from manufacturer 'B'. The total gentamicin C content of the samples from manufacturer A also seem to be higher than those from B, though since sulphate levels were not determined it cannot be stated categorically. The Chinese material showed a marked reduction in the total gentamicin C content, and this was confirmed by TLC which revealed very high minor component levels. Table 3.2 can be converted to component ratios by dividing by the respective gentamicin C totals to give Table 3.3.

Table 3.3. Component Ratios of the Commercial Samples

Sample	% C_{1a}	% C_2	% C_1
A1	26.0	38.9	35.1
A2	25.3	38.9	35.8
A3	24.9	39.3	35.7
B1	29.1	38.7	32.2
B2	26.3	42.8	30.9
B3	28.8	40.5	30.8
Chinese	24.9	49.1	26.0

The samples of manufacturer A show very similar component profiles illustrating low batch variability. The samples from manufacturer B do not exhibit such small variation but are probably within acceptable limits. There is obviously some difference between the two sources but this is unlikely to provoke widely differing clinical responses. The Chinese material, however, is markedly different from the other two sources, with a very high gentamicin C_2 content and low C_1 content. If the toxicity profiles of the components are different then the Chinese material would be likely to have differing properties to the two other sets of materials.

When a Hypersil column was used instead of a Spherisorb column, using otherwise identical conditions, the order of retention was altered with the gentamicin C_1 peak eluting before the C_{1a} and C_2 peaks. An extra peak was also observed to be partially separated from the gentamicin C_2 peak and this was thought to be due to gentamicin C_{2a} . This was reported to be separated by Freeman⁸² who also used a Hypersil column. A chromatogram of the retention of the gentamicin components on the Hypersil column is given in Figure 3.10. A full component analysis was not performed on this column because no standard for gentamicin C_{2a} was available and also because of the absence of a baseline separation between the gentamicin C_{2a} and C_2 components. However an estimation of the percentages of gentamicin C_2 and C_{2a} was made by reference to the component ratios already found for A1 and B1. The approximate levels are given in Table 3.4.

Figure 3.10. HPLC Chromatogram of Gentamicin Components
on a Hypersil Column

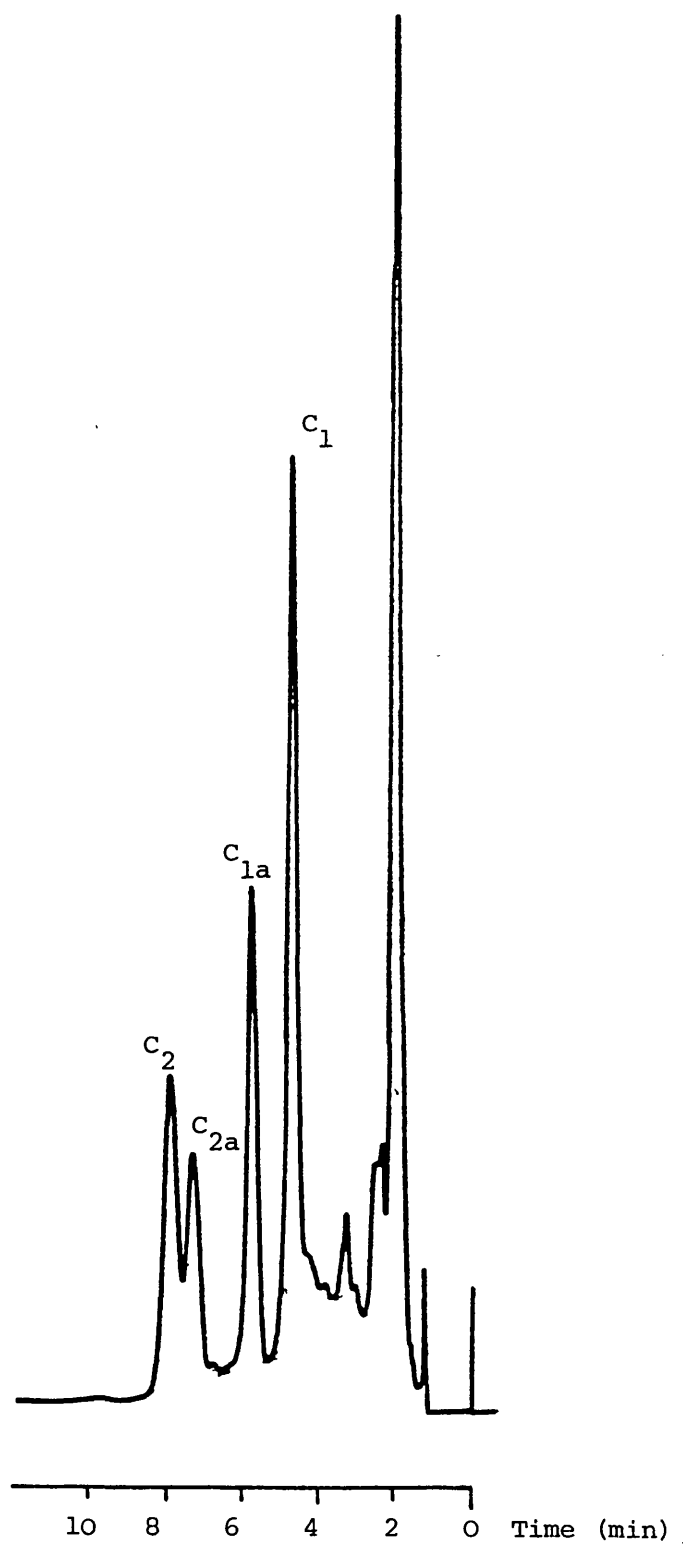


Table 3.4. Gentamicin C_2 and C_{2a} levels in commercial samples

Sample	(%) C_2	(%) C_{2a}
A1	26	13
B1	25	13
A4	25	11
B4	28	12
Chinese	24	20
Italian	33	12

The relative composition of samples from sources A and B are similar to each other, but quite different from the other two sources. The Chinese material had a very high gentamicin C_{2a} content especially in comparison to the C_2 level. Similar values have been reported by White⁷⁹ in commercial preparations though none with the same high ratio of C_{2a} to C_2 . The Italian sample, conversely, has a lower C_{2a} to C_2 ratio than that of the A/B sources. This system is thus useful for estimating the content of gentamicin C_{2a} in commercial samples which is difficult to achieve using other methods.

Assay of Gentamicin Injection

Two ampoules of the same batch of 40 mg/ml gentamicin injection were analysed for gentamicin C component levels. The calibration figures of the undecylamine peak height ratio were used to calculate levels from the equation:

$$\% \text{ component} = \frac{\text{ratio} - \text{intercept}}{\text{slope}} \times 0.99 \times \frac{a}{1.1} \times F_1$$

where a has the same value as used previously

F_1 is weight factor of the component solutions.

The 0.99 figure in the equation refers to an estimated 1% water content in the standards because the components were lyophilised immediately prior to solution preparation. A total of four determinations on each of the ampoules was performed, and as no significant difference was detected the results were combined. These results are given in Table 3.5 with component percentages of total C component given below that of the calculated percentage in the injection.

Table 3.5. Component levels in gentamicin injection

	Gent C _{1a}	Gent C ₂	Gent C ₁	C _{total}	Stand. Deviation
% calculated	21.2	49.9	34.5	105.6	2.04
Component ratio	20.1	47.3	32.6	100	-

The determination of total component composition for injections of gentamicin is by potency test in the BP³⁰ and these limits, related by logarithms, cannot satisfactorily be applied to this HPLC assay. The component ratio volumes reveal a very high gentamicin C₂ content and low gentamicin C_{1a} levels when compared to the values in Table 3.3. It is

another indication of the potential for variation in the composition of gentamicin C samples available commercially.

The HPLC assay used in this quantitative determination of commercial samples proved to be very useful in discovering the major component ratios, although definition of total gentamicin C content in the samples was not as satisfactory, primarily because of the lack of major components in a pure state. The use of undecylamine as internal standard was successful, although no clear advantage of peak area measurement was observed which may be related to the lack of a total baseline separation of the gentamicin C_{1a} and C₂ components. The levels of major components calculated were similar to those reported in the literature and conformed to the FDA regulations³¹ regarding component composition. The disadvantage of the HPLC system used, in not separating gentamicin C_{2a} was not felt to be a major drawback. This was because column responses would have to be estimated in the absence of a C_{2a} standard and the lack of literature information on the component would signify uncertainty as to whether high or low levels were deleterious to the sample.

3.3.2 High-performance Liquid Chromatographic Analysis of Gentamicin in Blood Plasma

In order to assess HPLC as an assay technique in the determination of gentamicin in blood plasma, a suitable separation method of the drug from constituents of plasma was required. Various methods from the literature as well as developed

methods were investigated. Certain requirements were set to ensure relevant clinical performance. The most important aspect of the techniques used was the precision of the method. Crude precision tests were performed by comparing peak heights for replicate assays to give an indication of the variability and this was reinforced by studying the steps involved in each method. The sensitivity of the method was also considered to be very important, especially since it is related to precision at low concentrations where small measurements of peak height have to be made. An arbitrary scale of peak heights was devised to compare the assay methods investigated with the assay method finally chosen, in terms of sensitivity. Specific recovery experiments were not performed because this was considered a less important aspect of the method. One hundred per cent recoveries were not sought but the variability of the recovery had to be low, and the sensitivity high. Estimated recoveries for each method are given, calculated from the recovery experiment on the chosen assay method and the sensitivity value with respect to that method. Qualitative aspects of the methods were also assessed in terms of ease of use, number of transfers and amount of labour required

The three quantitative parameters used to compare the different separation methods are given in Table 3.6.

Table 3.6. Performance of Assay Methods

Method	Concentration ($\mu\text{g/ml}$)	Number	Precision (%)	Sensitivity (%)	Recovery (%)
Silica Column	10	4	19	130	106
Acetonitrile Precipitation	10	3	32	108	73
TCA Precipitation / Ether extraction	(2 ml) 10 (5 ml) 10	4 6	19 8	85 135	23 37
TCA Precipitation/No Extraction (Chosen Method)	7.5	4	8	100	106

Extraction onto a Silica Column

This method summarised in Figure 3.11 was used by Maitra et al.²²³ to separate gentamicin from the plasma before derivatisation with OPA. Table 3.6 shows that although the sensitivity and recovery were good the precision was low. This lack of reproducibility was also described by Marples²³³ and may have arisen from differing adsorption of the gentamicin to the silica column. Delaney et al.¹⁷⁹ used silicic acid as a suspension to extract gentamicin, but this method was not examined in this study. A Sephadex column was utilised in a similar manner as in Figure 3.11, but even lower precision was achieved. The methodology was promising in terms of sensitivity and ease of use but the lack of precision appeared insurmountable.

Acetonitrile Precipitation and Ethyl Acetate Extraction

The methods of Bäck²³¹ and Haughey²³² for isolating aminoglycosides were assessed for suitability as a clinical assay. The extraction procedures for each are comparable with Bäck's method shown in Figure 3.12²³¹. Haughey's extraction procedure differs only in the quantities of solvents used and the final extraction being into isopropanol instead of ethyl acetate²³². With ethyl acetate extraction only one peak in the position of gentamicin C₁ was distinguishable by HPLC, though all three components were confirmed to be present, by evaporating the ethyl acetate and redissolving in methanol. It seems most likely that the high solvent strength of the ethyl

Figure 3.11. Extraction of Gentamicin onto a Silica Gel Column.

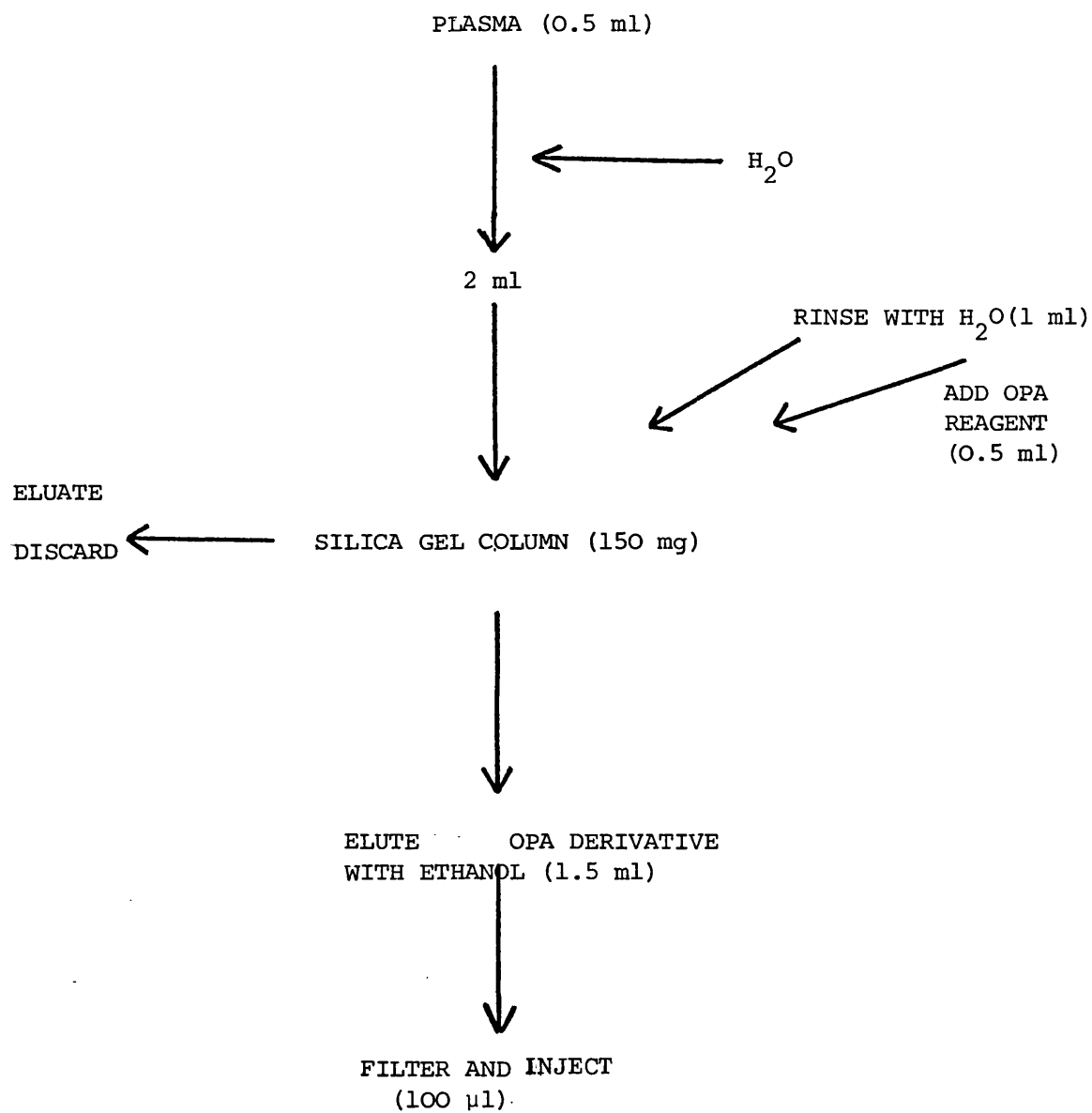
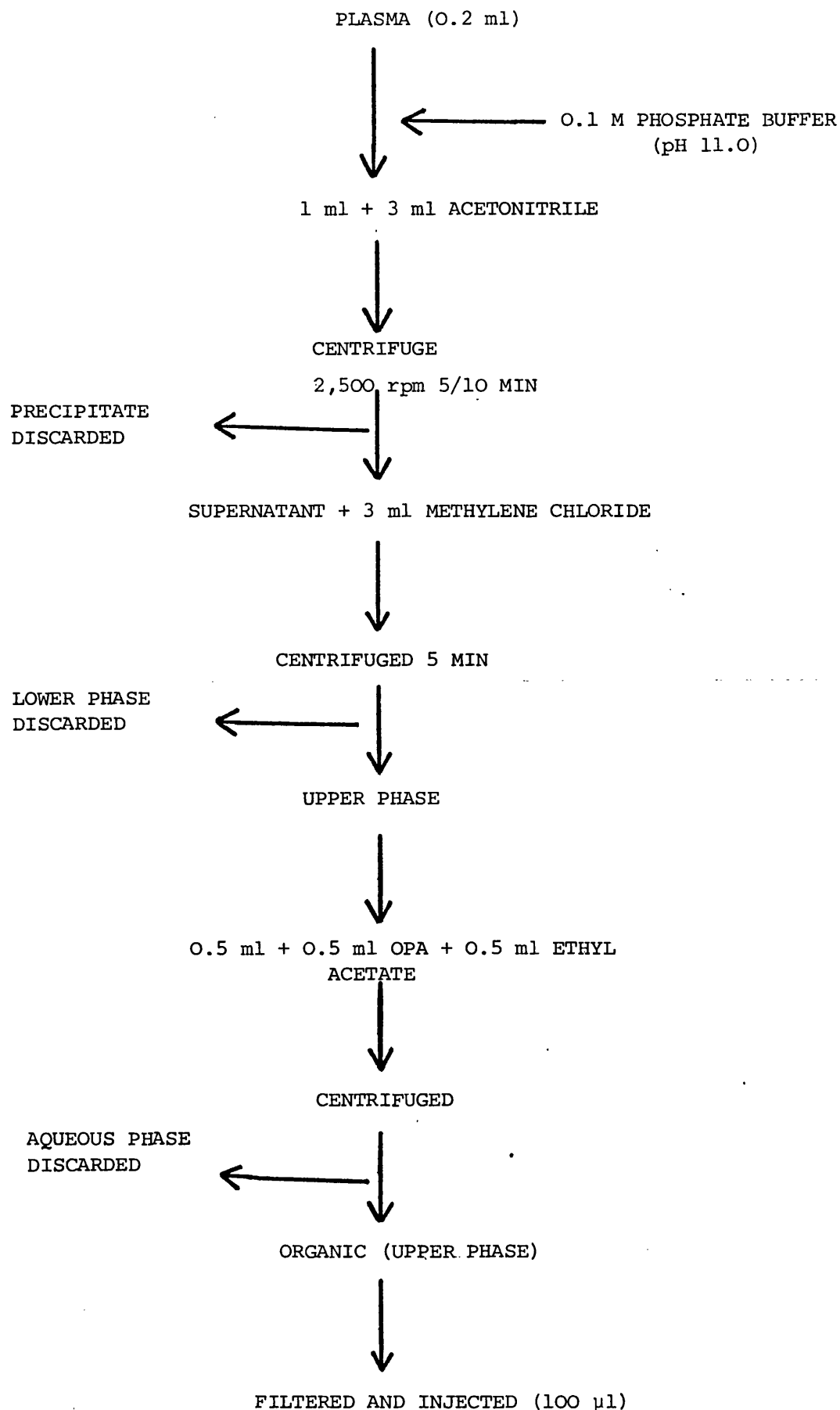


Figure 3.12. Acetonitrile Protein Precipitation, Ethyl Acetate Extraction.

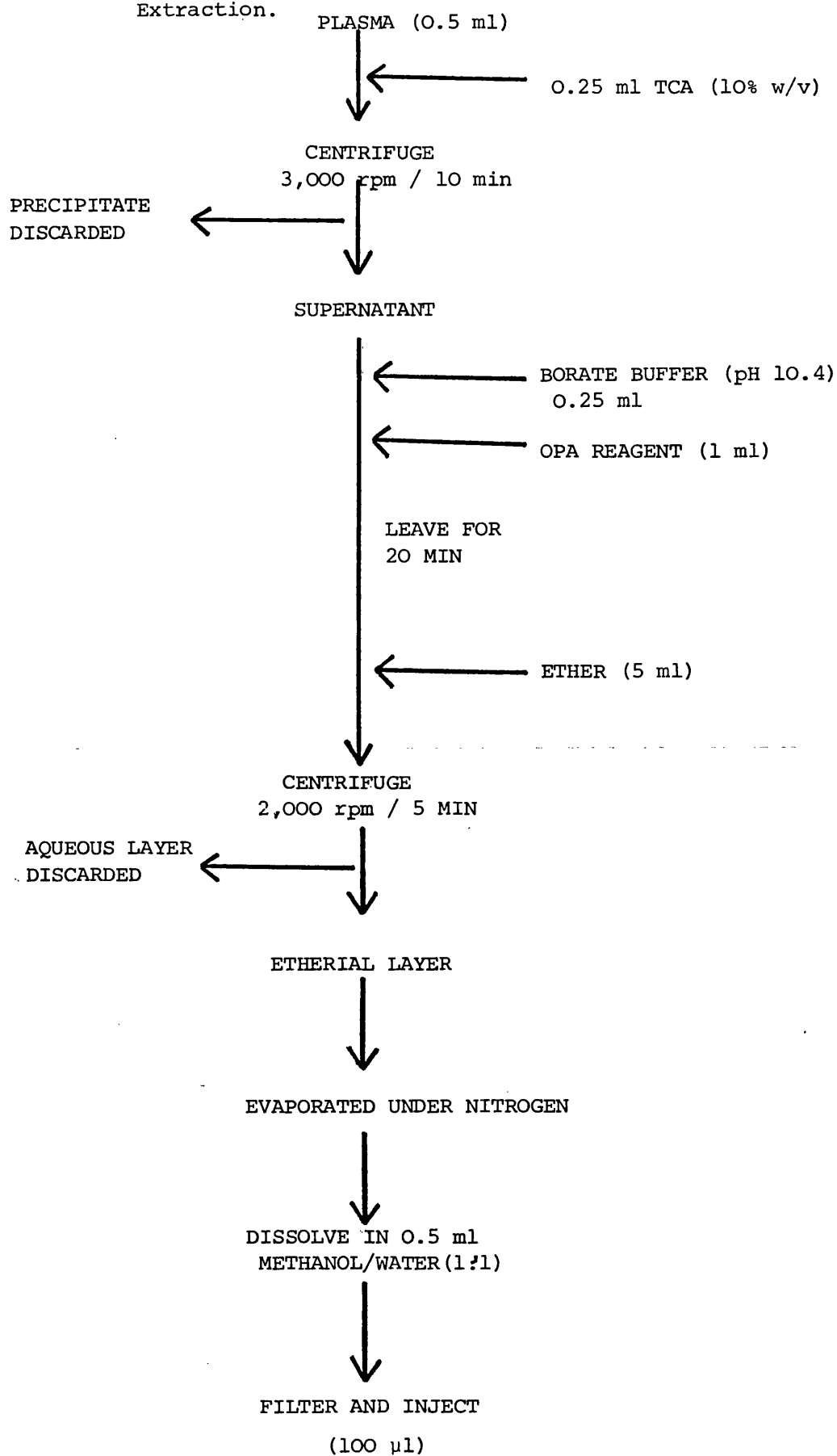


acetate eluted the components more rapidly and caused the earlier eluting C_{1a} and C_2 components to merge with highly fluorescent material near the solvent front. The use of isopropanol for the final extraction produced peaks for all components, but the high salt content of the aqueous solution, required for efficient partition, into this solvent, led to excessive extraction of other fluorescent material into the organic layer. The phosphate buffer used in both assays was precipitated by the acetonitrile and may have interfered with the protein precipitation. Precipitation with acetonitrile did not seem to be complete because of the cloudy supernatant produced. Extraction with methylene chloride was difficult as an emulsion formed easily with too much mixing. Spiking of the sample at each stage in the procedure showed a significant loss at the methylene chloride extraction. This may account for the sensitivity not being as high as expected and a high coefficient of variation for three samples of 10 $\mu\text{g/ml}$ concentration (Table 3.6). The entire method was thought to be too labour intensive and unsuitable for clinical determinations. The large number of transfers suggests that a high precision would be very difficult to achieve.

Trichloroacetic Acid Precipitation and Diethyl Ether Extraction

Despite the many disadvantages of the extraction method it is desirable because of the removal of interfering substances and also to protect the HPLC column from irreversibly

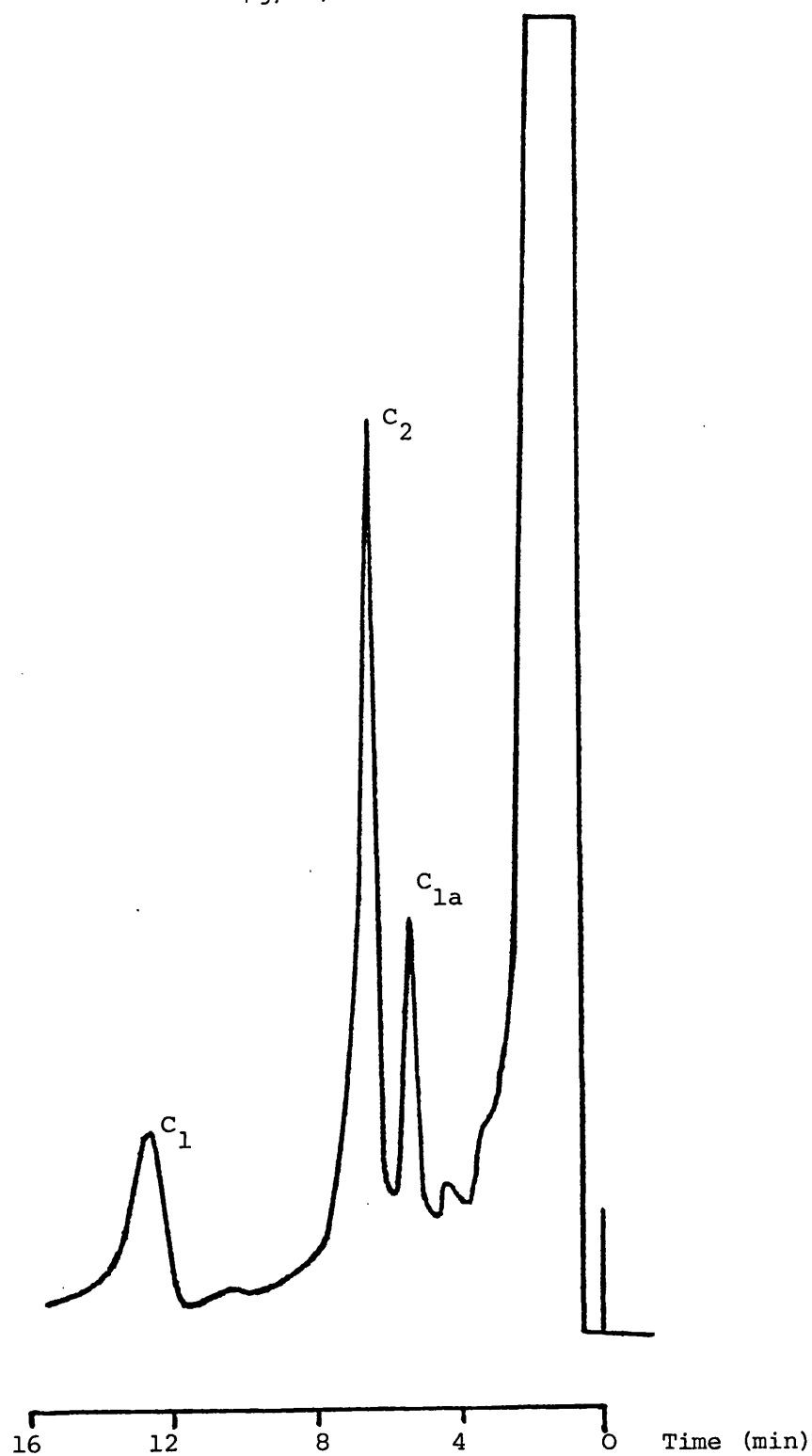
Figure 3.13. Trichloroacetic Acid Precipitation, Diethyl Ether Extraction.



bound compounds. Thus a superior protein precipitation technique was required, coupled with a reproducible extraction method. The developed method is shown in Figure 3.13. Trichloroacetic acid (TCA) is a well known precipitating agent^{217,218} and has been used with gentamicin in a previous study by Kraisintu⁷⁷.

The TCA was dissolved in 80% v/v methanol because it enhanced precipitation over the aqueous analogue and produced a clear supernatant. Prior to reaction with OPA, borate buffer (pH 10.4) was added to neutralise excess TCA and maintain an alkaline pH. The reaction was allowed to continue for only twenty minutes in order to restrict total assay time to a minimum. The ether added was easily separated from the aqueous layer and evaporated by nitrogen within five minutes. Initially only 2ml of ether was used to extract the gentamicin derivatives but this gave a low recovery and a large coefficient of variation at 10 µg/ml (Table 3.6). The reproducibility of ether extraction was monitored using aqueous solutions of gentamicin and larger volumes of ether were found to be required. The 5 ml of ether finally adopted increased the recovery, with only a 8% coefficient of variation for six samples at 10 µg/ml (Table 3.6). This method was thought suitable for clinical use in terms of ease of handling and performance. However the ether extraction seemed to accumulate other constituents of the plasma as signified by the large, highly fluorescent peak at the beginning of the chromatogram (Figure 3.14). Thus the ether extraction was of questionable value in protecting the column. The extraction step was also thought to be susceptible

Figure 3.14. HPLC Chromatogram of Gentamicin in plasma using
TCA precipitation and Ether Extraction (Gentamicin
10 $\mu\text{g/ml}$)



to environmental changes and interday precision may have suffered.

Trichloroacetic Acid Precipitation with No Extraction

This was a modification of the scheme shown in Figure 3.13 with the extraction procedure omitted and the volume of reagents reduced to a minimum to achieve adequate sensitivity. The method developed for plasma assays was as shown in Figure 3.15. Levels as low as 0.5 µg/ml could be determined although the precision was poor at this concentration. The average percentage recovery of the components using this method was 106% as determined by comparison with an aqueous calibration. The slight excess was probably due to the exact volume of supernatant produced being unknown, but taken as 0.4 ml. A good recovery is thus observed, suggesting that if plasma binding of gentamicin does occur, it does not interfere with the assay. Undecylamine internal standard had to be added after protein precipitation because of extensive protein binding of the compound if added before. This method required about 1½ hours for a single assay but involved few manipulations and thus several assays could be performed concurrently. In practice one worker could complete twenty-five single assays in a day. A typical chromatogram of a 2.5µg/ml sample is illustrated in Figure 3.16.

Figure 3.15. Trichloroacetic Acid Precipitation, No Extraction.

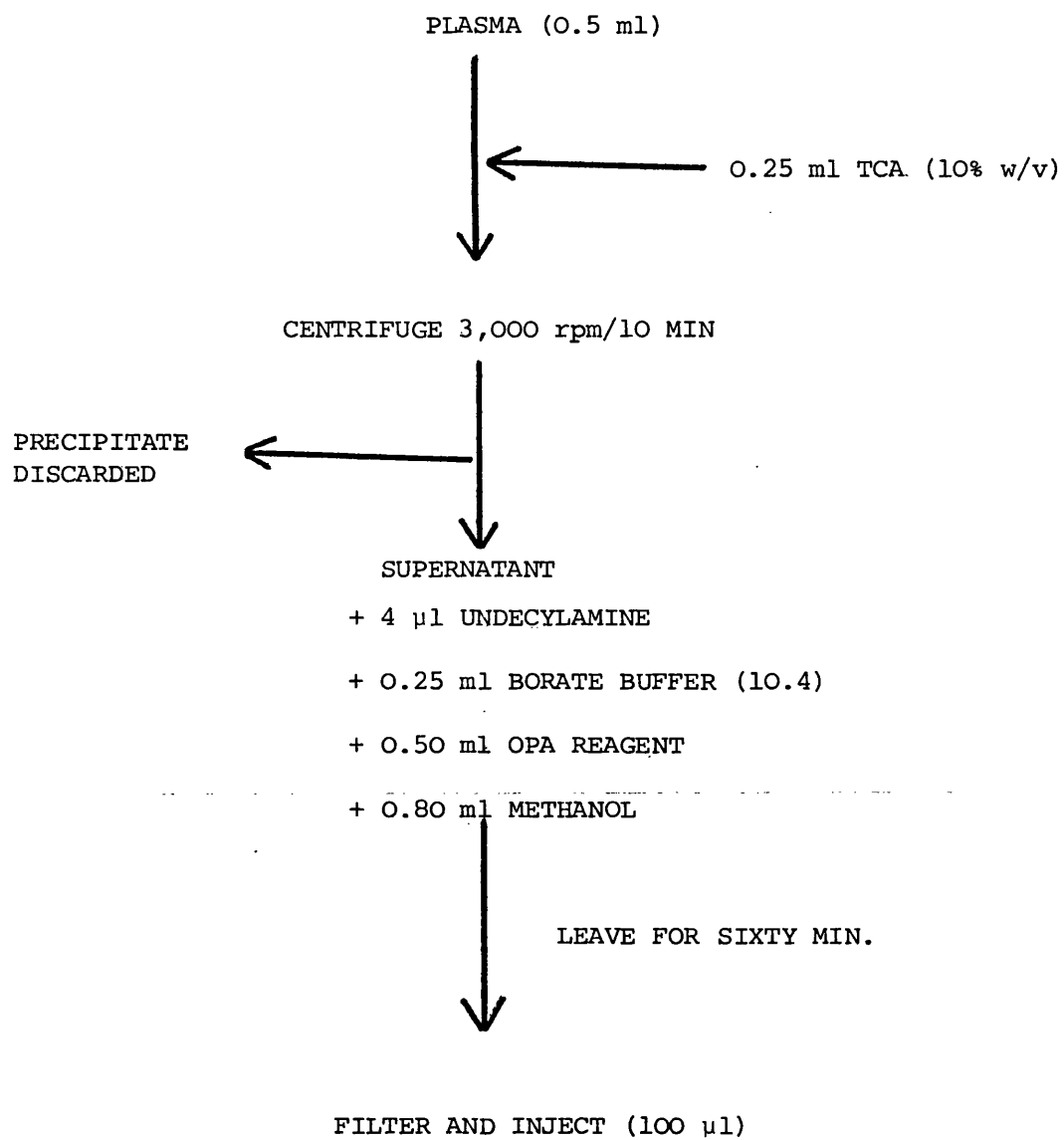
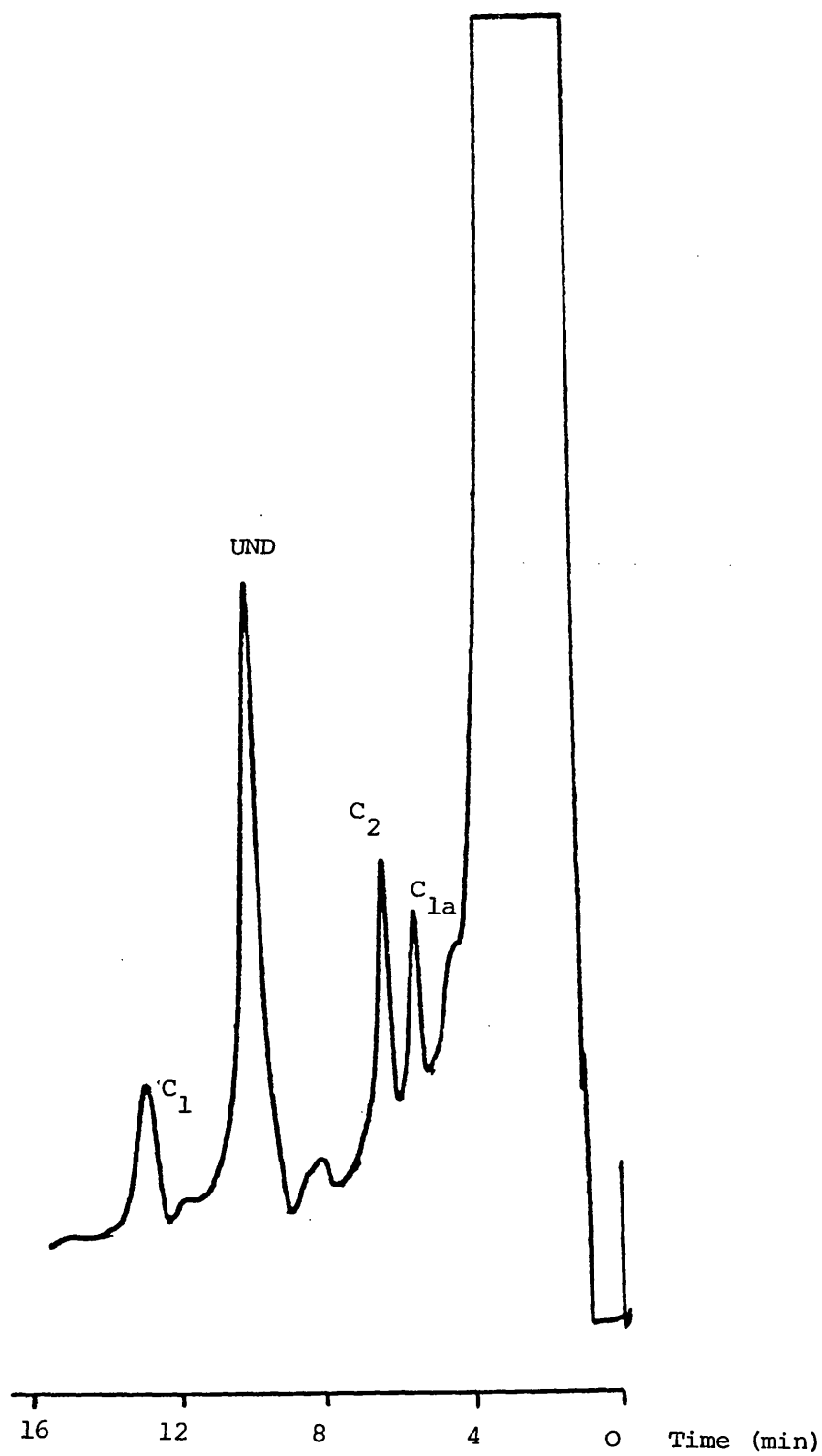


Figure 3.16. HPLC Chromatogram of Gentamicin in plasma
(2.5 µg/ml) using TCA precipitation and
no extraction



Calibration for Plasma Assay

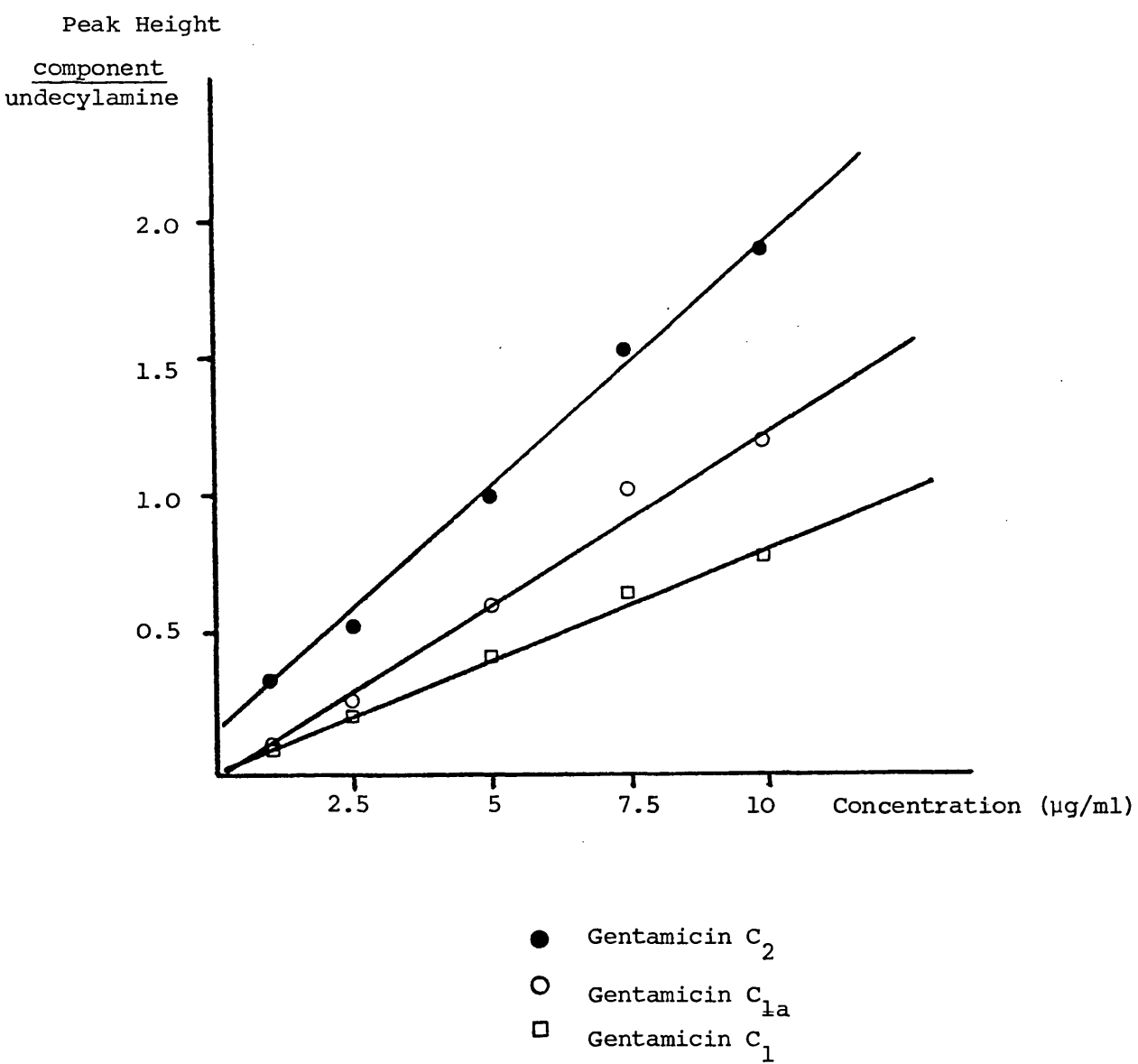
A series of calibrations in the range of 1 - 10 µg/ml were run with the spiked samples. A plot of one such calibration is illustrated in Figure 3.17. The parameters utilised for measurement were the peak height ratio of component to internal standard, and just the peak height of component without reference to standard. The latter was used in case the undecylamine showed variation in response due to unusual plasma binding. The mean of eight calibrations was taken to provide regression values as shown in Table 3.7.

Table 3.7. Mean values of calibrations

	C _{1a}	C ₂	C ₁	C _{1a} /UND	C ₂ /UND	C ₁ /UND
Slope	8.750	13.250	6.570	0.1038	0.1697	0.0768
Intercept	6.062	21.882	3.856	0.0680	0.2413	0.0587
% C.V.	10.3	7.5	6.5	8.0	7.3	6.5

The sloping baseline of the chromatogram meant that peak height measurement offered greater accuracy than peak area measurement. The high intercept observed for the gentamicin C₂ was consistent and must have been due to an underlying peak which was undetected. The calibrations run on separate days indicated a stable system and so during the analysis of clinical samples, calibrations were not made each day. Instead to test the relative daily column response, standard spiked

Figure 3.17. Calibration of Gentamicin Components in plasma
using undecylamine as internal standard



plasma samples of 2.5 and 7.5 $\mu\text{g/ml}$ were injected after every two patient samples. The mean of all of the standards used in a day was compared with the expected value for all components and both sets of calibrations. The discrepancy of the observed and expected values was converted to a factor to adjust all of the days samples to the calibration figures of Table 3.7. This procedure saved time in performing a single calibration and was more flexible in that the columns response over a whole day was averaged. The mean of all of the factors used on the nine days of plasma sample assays are given in Table 3.8.

Table 3.8. Mean of factors used to correct calibration

	C_{1a}	C_2	C_1	C_{1a}/UND	C_2/UND	C_1/UND
\bar{x}	1.051	0.811	1.066	1.580	1.154	1.495
% CV	13.2	21.3	7.7	12.8	16.9	9.3

The factors used are seen to have varied significantly during the sample analysis, with the gentamicin C_2 component experiencing large changes. In relation to the other two components it had decreased in size, although the reason for this is unclear. It is also evident that the undecylamine peak height was reduced in respect to the gentamicin components from the calibrations. A cause may have been increased protein binding even though the methodology was constant throughout. The intra-assay and inter-assay variance of the injected standards were calculated to assess the viability of the method, and of the use of factors.

Table 3.9. Inter-assay Variance for both calibrations

Conc.	C _{1a}	C ₂	C ₁	C _{Total}	C _{1a} UND	C ₂ UND	C ₁ UND	C _{Total} UND	Mean C _{Total}
2.5 µg/ml	0.702	0.697	0.910	2.31	0.777	0.851	1.021	2.65	2.48
% CV	10.5	13.0	11.2	11.6	14.6	14.3	19.8	17.2	16.0
Expected (µg/ml)	0.682	0.818	1.000	2.50	0.682	0.818	1.000	2.50	2.50
7.5 µg/ml	1.936	2.602	2.978	7.520	1.802	2.334	2.886	7.02	7.27
% CV	5.7	8.3	6.2	5.3	9.2	10.1	11.5	9.7	8.7
Expected (µg/ml)	2.045	2.454	3.000	7.50	2.045	2.454	3.000	7.50	7.50

The intra-assay coefficient of variation for four samples of 2.5 and 7.5 µg/ml was 5.1% and 8.3% respectively. The coefficient of variation of the interassay study of twenty determinations analysed on five consecutive days is given in Table 3.9, for both sets of calibrations. These figures were taken from the standards used and thus also include a measure of the precision throughout the day.

The interday assay figures reflect the variation in response of the system, with the lower concentration range especially susceptible. The undecylamine when used as an internal standard only added to the variation and this was probably related to the protein binding effect. Table 3.9 also shows the expected concentration of the individual components calculated from composition of standard gentamicin solution used. There is no apparent trend between the observed and expected concentrations except that the gentamicin C₁ component is the most accurately defined. This is not unexpected because unlike the other two components the gentamicin C₁ peak is not on the tail of the solvent front peak. The percentage accuracy with 95% confidence intervals is given in Table 3.10, calculated from the values obtained in the precision studies.

Table 3.10 Accuracy of assay method

	Strength (µg/ml)	% Accuracy	Confidence Ints.
Interassay	2.5	99	± 7.5
(n=20)	7.5	97	± 4.1
Intra-assay	2.5	97	± 7.1
(n=4)	7.5	98	±11.5

The accuracy in both cases is good and further justifies the use of standards instead of daily calibrations. The factors which are calculated from these standards can adjust the calibration to optimise the accuracy. This compensates for the inherently poor precision which may be due to column instability caused by the elution of methanol at the end of each day, to remove a build-up of bound material.

Assay of Patient Blood Plasma Samples

To assess the performance of this HPLC assay a total of sixty-four samples of plasma, from five patients receiving gentamicin therapy, were assayed for gentamicin. The determinations were in duplicate, assayed blind, and calculated using peak height measurement with and without internal standard. The results obtained were compared with those found by microbiological assay, supplied by Dr. D. Scott, The General Hospital, Birmingham. Only fifty-seven of the readings determined could be compared with those found by microbiological assay. The regression of the values found by HPLC on those of the microbiological assay is shown in Figure 3.18. The value of total regression analysis and individual patient regression is given in Table 3.11. The slopes are all similar in magnitude though variation is seen between patients. The different slopes obtained for the various patients may be a measure of the variation in column response or due to the differing composition of the individual patients plasma.

Figure 3.18. Regression of HPLC Assay on Microbiological
Assay (n = 57)

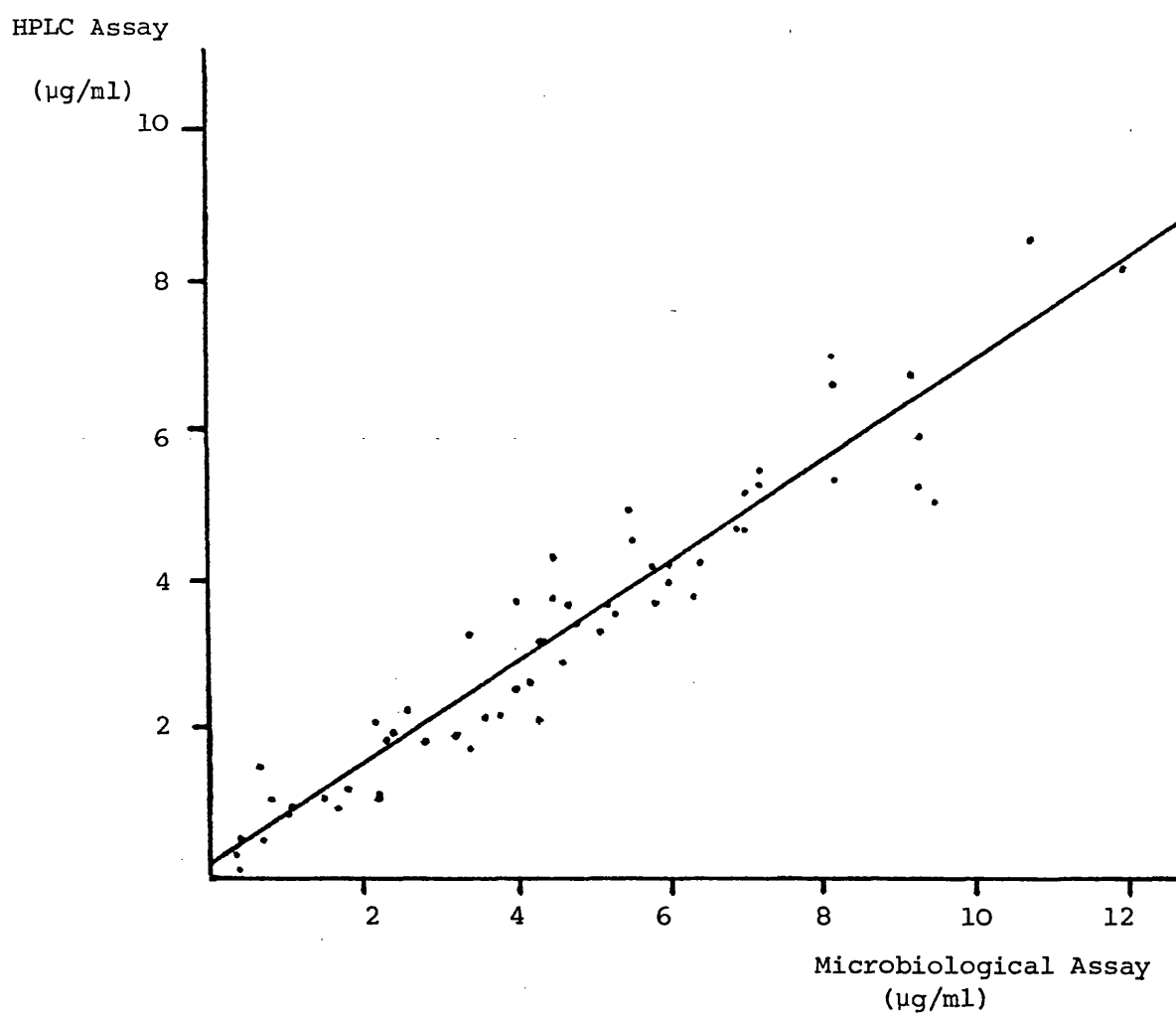


Table 3.11. Regression of HPLC on Microbiological method

Patient	r	Slope	Intercept	n
Total	0.958	0.669 ¹	0.177	57
Patient A	0.978	0.746 ¹	-0.461	11
Patient B	0.978	0.761 ¹	0.048	13
Patient C	0.971	0.611 ¹	0.363	13
Patient D	0.964	0.691 ¹	0.129	13
Patient E	0.917	0.497 ¹	0.936	7

1. $p < 0.01$, Students t-test

The total correlation coefficient (r) is significant ($p < 0.01$) and suggests that the HPLC assay is comparable to the microbiological assay. However the slopes of the regression are significantly different from unity in all cases ($p < 0.01$) as indeed is the total slope.

Various explanations can be proposed for the slopes not equalling unity. Firstly the samples were old and had been stored at c -20°C for about four years. In this time the samples may have deteriorated with gentamicin binding irreversibly either to plasma constituents, or to the glass vials the plasma was stored in. Secondly, if high concentrations of penicillin were present, the well documented inactivation of gentamicin may have occurred¹³⁰⁻¹³⁸. Lastly, it could be an indication of a methodology difference due either to the different properties assayed or caused by a difference in standards.

It was observed that freezing the plasma, after spiking with gentamicin standard solution, decreased the peak height of the components, as measured by HPLC. This may have arisen from adsorption of gentamicin, but standards used for the calibration were stored frozen before use to overcome this loss. No significant loss of gentamicin was observed for frozen samples in the short term (seven days). However study of the storage characteristics up to 3 months produced results too variable to quantitate, but revealed no apparent trend in deterioration. One study in the literature²⁴², using a bioassay found no loss on storage for two days in plastic or glass containers at -20°C , 4°C and 25°C . However decrease in the gentamicin level was observed when carbenicillin was present ($> 500\text{ }\mu\text{g/ml}$) at all temperatures. A study²⁰⁷ comparing the EMIT method with the bioassay found a relationship with a correlation of 0.96 and slope of 0.91 when assayed on the day of collection. However when specimens were stored at -60°C for one to sixty three days before EMIT measurement the correlation was 0.95 with a slope of 0.69. This compares with a slope of 0.67 for the similar regression of HPLC on the microbiological results observed here. The authors of the paper ascribed the loss of gentamicin to a general phenomenon on freezing and not to a difference in the two techniques. However some other comparisons in the literature also show similar differences. In a paper by Mannisto¹⁷⁷ the slope of EMIT results regressing on the bioassay is 0.67 ($r = 0.92$, $n = 62$). Here the bioassay was performed on the day of collection and the EMIT methods on samples stored for 1 - 2 months at -27°C .

However the slope of the EMIT results regressing on a fluoro-immunoassay was only 0.78 ($r = 0.86$, $n = 62$) where the samples were stored identically. This variation in the calibration of different methods is also shown in another paper²¹² where the regression of a fluoroimmunoassay on a bioassay for amikacin has a slope of 0.69 ($r = 0.81$, $n = 160$). This difference was attributed to a difference in standards, and although this is plausible for radioimmunoassays it is less likely here.

Studies in the literature do not report many direct comparisons of the EMIT method with a bioassay, and although many papers of HPLC methods^{221,227,231,233} have reported good correlations with slopes close to unity, in respect to the bioassay, the discrepancies noted here do cause concern. This was highlighted in a paper by Faber *et al.*²⁴³ where the bioassay was found not to agree with results determined by HPLC. A comparison showed the HPLC method to agree with the EMIT method and to be highly specific, especially in the presence of other antibiotics. It was suggested that, with gentamicin present, the penicillins were not totally inactivated by penicillinase and gave falsely high readings for the bioassay.

In this study the difference between the two methods may be explained in a number of ways, but the most feasible is that of a loss on freezing as noted in other papers.

The time course of the gentamicin concentration in plasma can be plotted though more results would be required for a complete analysis. The decay curve is typical for aminoglycoside

administration and is described by a two compartment open model which accounts for tissue accumulation of gentamicin¹¹⁸. However for simplicity, and insufficient time points it is here assumed to follow a one compartment model with the equation¹¹⁹:

$$C = Be^{-\beta t}$$

where C is concentration in plasma

B is extrapolated concentration at $t = 0$

β is apparent elimination rate constant

t is time after administration

For patient B a time course curve is shown in Figure 3.19. This can be translated to a linear format by taking logarithms.

$$\ln C = \ln B - \beta t$$

The plasma half life ($t_{1/2}$) and apparent volume of distribution (V_D) can be calculated from:

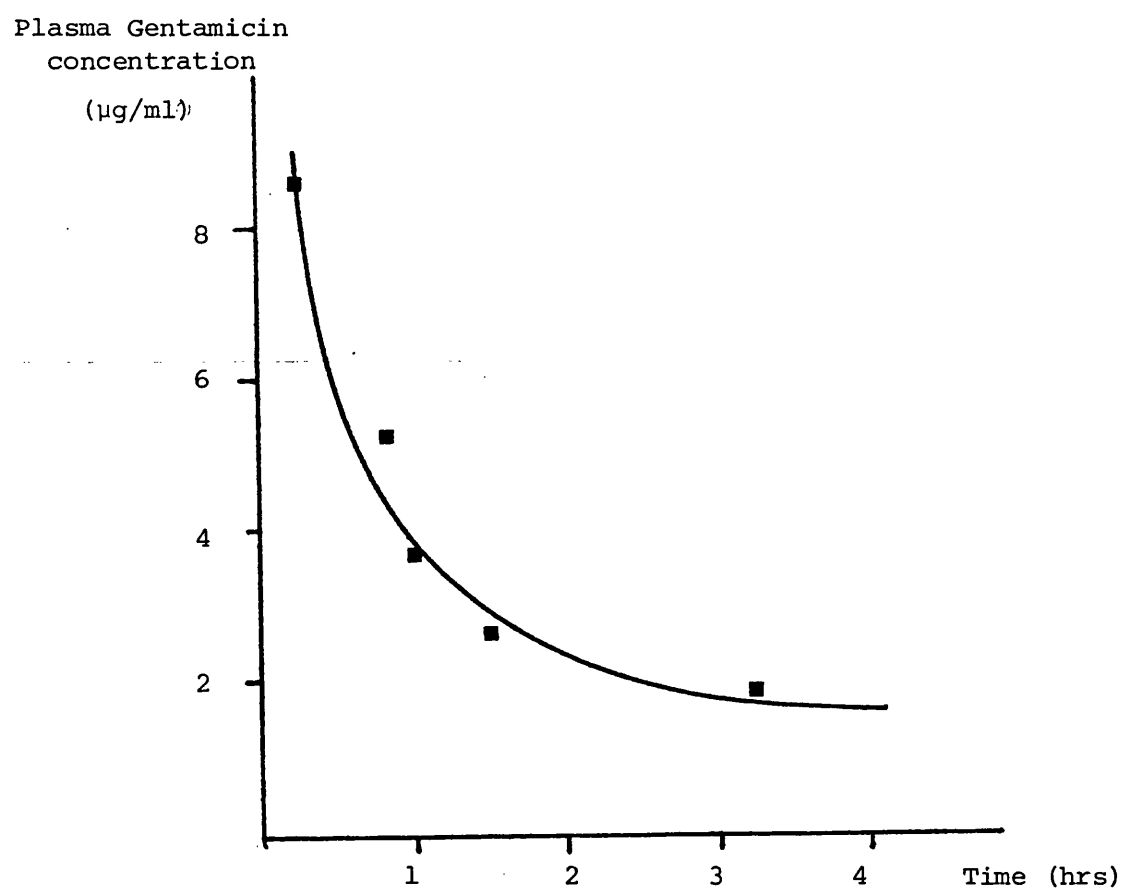
$$t_{1/2} = \frac{0.693}{-\beta}$$

and

$$V_D = \frac{\text{Dose}}{B}$$

From the plasma concentrations determined by the assay, estimates of these pharmacokinetic parameters were made as defined in Table 3.12. As slopes were estimated from three

Figure 3.19. Time Course of Gentamicin in plasma (Patient B)



points, in some cases, the results were not regarded as accurate.

Table 3.12. Calculation of pharmacokinetic parameters

Patient	n	r	$t_{1/2}$ (hr)	V_D (l)	V_D (%)
A	4	0.994	1.65	11.82	24
A	3	0.922	1.31	2.60	5
B	4	0.988	2.75	21.06	36
B	5	0.911	1.45	10.94	19
C	3	1.000	2.54	20.39	25
C	3	1.000	2.17	20.87	28
C	3	0.996	4.04	12.87	16
D	7	0.948	1.31	19.25	31
D	3	0.999	1.59	12.52	20
E	6	0.966	3.23	23.05	33

The values reflect the variation due to the small sample size and the use of the one compartment model. The average mean half life in the patients, using HPLC, was 2.20 ± 0.66 compared to 2.44 ± 1.02 found by the microbiological assay. These figures compare well with the expected value of about two hours for individuals with normal renal function¹⁰⁷. The apparent volume of distribution also varied due to the small sample size but the average of 23.7% is close to the 20% value reported for the aminoglycosides¹⁰⁷. With more readings for a single injection a more accurate estimation

of the pharmacokinetic parameters could be made. It does, however, illustrate the usefulness of determining the plasma concentrations for aminoglycosides. These values can be determined by any of the plasma assay techniques, yet only HPLC can offer the estimation of individual component parameters.

Using the peak height determinations of the individual components a plot of component concentration against time could be produced as in Figure 3.20 for patient D.

Assuming again, the one compartment model, the half-life and apparent volume of distribution can be calculated for the components and patients. These values are given in Tables 3.13 and 3.14. The volumes of distribution were calculated by assuming the gentamicin administered to be composed of

Table 3.13. Half life of components in patients

Patient	$t_{1/2}(C_{1a})$	$t_{1/2}(C_2)$	$t_{1/2}(C_1)$
A	1.99	1.51	1.99
A	1.56	0.73	1.36
B	3.82	1.73	2.11
B	1.81	1.61	1.09
C	4.42	2.65	1.80
C	3.31	1.54	1.64
C	4.25	3.42	3.35
D	1.88	1.37	1.18
D	2.54	1.16	1.71
E	5.58	3.66	2.22
\bar{x}	3.12	1.94	1.85
Stand. Dev.	1.37	0.97	0.65

Figure 3.20. Time Course of Gentamicin Components in plasma
(Patient D)

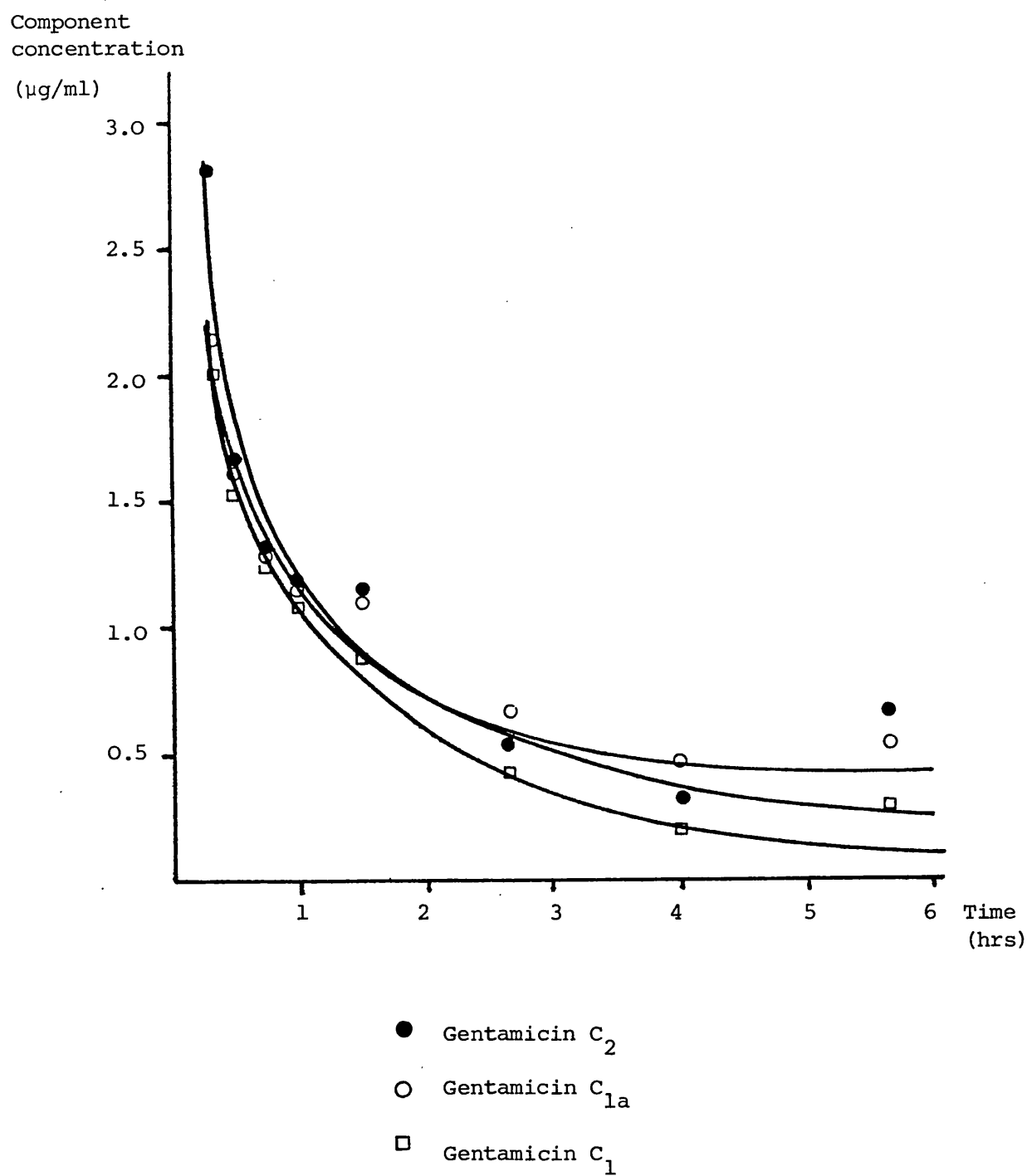


Table 3.14. Volume of Distribution of Components

Patient	V_D (litres)			V_D (%)		
	C_{1a}	C_2	C_1	C_{1a}	C_2	C_1
A	13.54	18.45	14.89	27.1	36.9	29.8
A	3.52	0.38	2.82	7.0	0.8	5.6
B	23.88	19.66	15.90	41.1	33.9	27.4
B	12.11	12.86	7.88	20.9	22.2	13.6
C	26.57	23.42	14.53	33.2	29.3	18.2
C	25.96	19.51	20.60	32.5	24.4	25.8
C	12.66	11.13	10.02	15.8	13.9	12.5
D	16.54	20.92	19.16	26.7	33.7	30.9
D	15.40	10.12	13.68	24.8	16.3	22.1
E	25.10	28.05	20.63	35.7	39.8	29.3
\bar{x}	17.53	16.45	14.01	26.48	25.12	21.52

the mean of the composition of samples A1 - B3 in the component analysis section because the composition of the injection was unknown. That is 26.7% C_{1a} , 39.9% C_2 and 33.4% C_1 . It is apparent from Table 3.13 that gentamicin C_{1a} has a longer half life than the other two components, even though the figures are only estimates. Riff and Jackson¹¹³ noted that patients with normal renal function could be divided into those with an accelerated response, those with a typical response and those with a damped response in terms of the gentamicin plasma concentration profile. They postulated that the difference

could be due to a reversible binding of gentamicin by constituents of blood, possibly the red blood cells. Such binding may also account for the relative differences in half life of the individual components.

Lachatre et al.¹⁵¹ in experiments on rabbits found that the cumulative urinary elimination of gentamicin C_{1a} was significantly lower than for the other two components. This was partly explained by a greater renal accumulation of the component. The volumes of distribution calculated for the components given in Table 3.14 are relatively similar with the gentamicin C_1 having the smallest volume. If gentamicin C_{1a} was bound to a larger extent in another compartment the volume of distribution should be larger. That this is not the case suggests the components behave in a comparable manner in terms of tissue distribution.

The HPLC method developed for the assay of gentamicin in plasma proved to have good accuracy with a high correlation with the microbiological assay. The difference between the two methods is relevant and the possible loss of gentamicin on freezing warrants investigation. The HPLC method was found to be useful in pharmacokinetic studies and pointed to possible differences in the handling of components which may be of importance in terms of relative toxicity.

CHAPTER 4

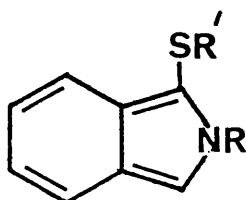
ORTHO PHTHALALDEHYDE REACTION IN RELATION TO GENTAMICIN

4.1 Introduction

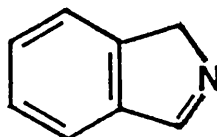
The ortho-phthalaldehyde reagent (OPA), described by Roth²³⁷ has proved to be extremely useful in the high-performance liquid chromatography (HPLC) of amines and amino acids²⁷⁹⁻²⁸⁵. The fast reaction with such compounds has made it an ideal reagent for both post- and pre-column derivatisation²³⁶. The fluorescent product exhibits an excitation maximum wavelength of 340 nm and an emission maximum at about 430 nm^{283,286-8}. An excitation maximum at 229 nm which is reportedly more intense than at 340 nm also exists²⁸². However problems of interference at the lower wavelength by constituents of plasma have been envisaged. The reagent used for derivatisation requires a thiol to be present with the dicarbonyl aromatic, before an amine is added²⁸⁹. If the amine is added before the thiol, the fluorescent yield is reduced, probably because of a different reaction pathway²⁸⁹. The nature of the thiol used can have a significant effect on the maximum emission wavelength, whilst the amine is less critical in this regard²⁸⁶. Alcohol has been reported to enhance the fluorescence produced, whilst a solvent such as chloroform can quench it^{281,286}. The pH of the reaction has also been discovered to be an important determinant of fluorescence yield and stability of the product^{283,286}. The fluorescence produced is diminished below pH 7.5 and boric acid as a buffer species is thought to promote greater stability than phosphate^{237,286}. The amine involved in the reaction can affect the stability of the product since bulky groups

adjacent to the amino function protect against decomposition^{289,290}

The structure of the fluorescent product was discovered to be that of a 1-alkylthio-2-alkyl substituted isoindole (4.I)²⁹¹



(4.I)



(4.II)

This structure was postulated by Simons after examination of the product by high resolution mass spectrometry, proton nuclear magnetic resonance and infra-red spectroscopy²⁹¹.

Mass spectrometry defined the molecular formula, whilst the infra-red failed to show adsorption bands due to carbonyl or sulphydryl groups, thus eliminating some structural possibilities. The proton NMR spectrum revealed a 2:1:2 proton split in the aromatic region which was in accord with the spectrum of isoindole²⁹²⁻³.

Isoindole is an unstable compound with a tendency to form the isoindolenine isomer (4.II) instead of the o-quinoid form^{293,296}. Substitution at the nitrogen is known to stabilise the isoindole structure²⁹³, and substitution of the thiol at the 1 position probably exerts a similar influence²⁸⁹. The extended conjugation of the molecule (4.I) is responsible for the observed spectral characteristics.

The fluorescent product of the OPA reaction undergoes photodecomposition, which may arise from intramolecular attack by the hydroxy group of the mercaptoethanol²⁸⁹, or to intermolecular attack by excess OPA²⁹⁰. The mechanism of both reactions could be by nucleophilic addition.

The gentamicin-o-phthalaldehyde derivatives when assayed by HPLC have shown different retention orders. Maitra²²³ and others^{231,233}, have reported the gentamicin C₁ derivative to elute first, followed by gentamicins C_{1a} and C₂. However, in the assays reported in Chapter 3 and in a previous study^{7,78}, the order of elution was gentamicin C_{1a}, C₂ with C₁ last to elute. Since nearly identical conditions to those of Maitra were used, it was important to determine the source of this variation, to ensure integrity of the assay procedure. The difference in retention order may have arisen from either distinct reaction products, or from a variation in column response to the derivatives. Before the latter could be fully investigated the former potential source of inequivalence had to be eliminated.

It is known that the OPA reagent will only react with primary amines to form the fluorescent product; secondary amines being unsuitable as substrates²⁹¹. The gentamicin C_{1a} and C₂ components each have four primary amino functions on each molecule, whilst gentamicin C₁ has only three. Thus if all groups were reacted to form the hydrophobic isoindole functions, the retention order on a reverse phase column

would be expected to be C_1 , C_{1a} then C_2 . However, if not all of the primary amino groups on the components reacted, perhaps for steric reasons, then the retention order would be C_{1a} , C_2 then C_1 , if an equal number on each component reacted.

The estimation of the extent of the reaction was believed to be best accomplished by use of proton NMR. Figure 4.1 illustrates the 60 MHz spectrum of o-phthalaldehyde (OPA) in chloroform. The aromatic protons are distinguished by a multiplet at $\delta 7.7$ to 8.3 , and the aldehyde protons as a singlet at $\delta 10.68$. It was thought that the reaction could be easily followed by the disappearance of the aldehyde protons, whilst the gentamicin concentration could be monitored by the C-methyl peak at $\delta 1.18$ common to all three of the gentamicins. However, Figure 4.2 shows the proton NMR spectrum of the reaction mixture equivalent to that used in the HPLC derivatisation method. It is clear that the aldehyde protons are no longer present, though a peak at $\delta 6.63$ may represent the same protons. The aromatic signals are also simplified to a single broadened peak at $\delta 7.53$. Triplets at $\delta 3.7$ and $\delta 2.7$ are due to the methylene groups of mercaptoethanol. This spectrum was thought to belong to a reactive species not described by Simons²⁸⁹, which raised the possibility that a different pathway to that proposed by him was occurring with the reaction mixture. The reaction mixture required another measure of the extent of depletion of OPA during reaction with amines, and thus an investigation of the nature of the reaction mixture was made, using NMR.

To confirm the results of the NMR experiment other methods

Figure 4.1. Proton (60 MHz) NMR spectrum of o-phthalaldehyde in chloroform.

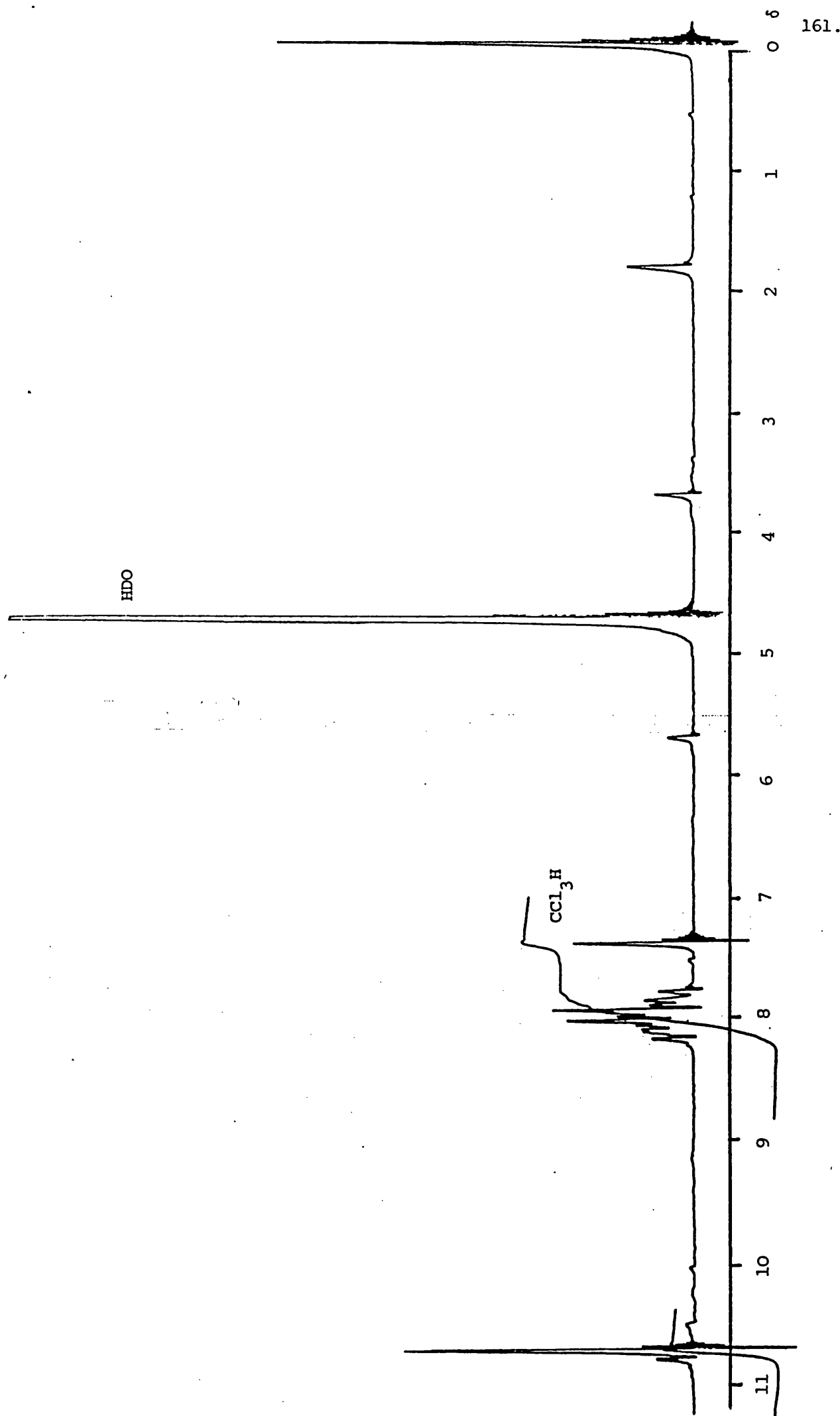
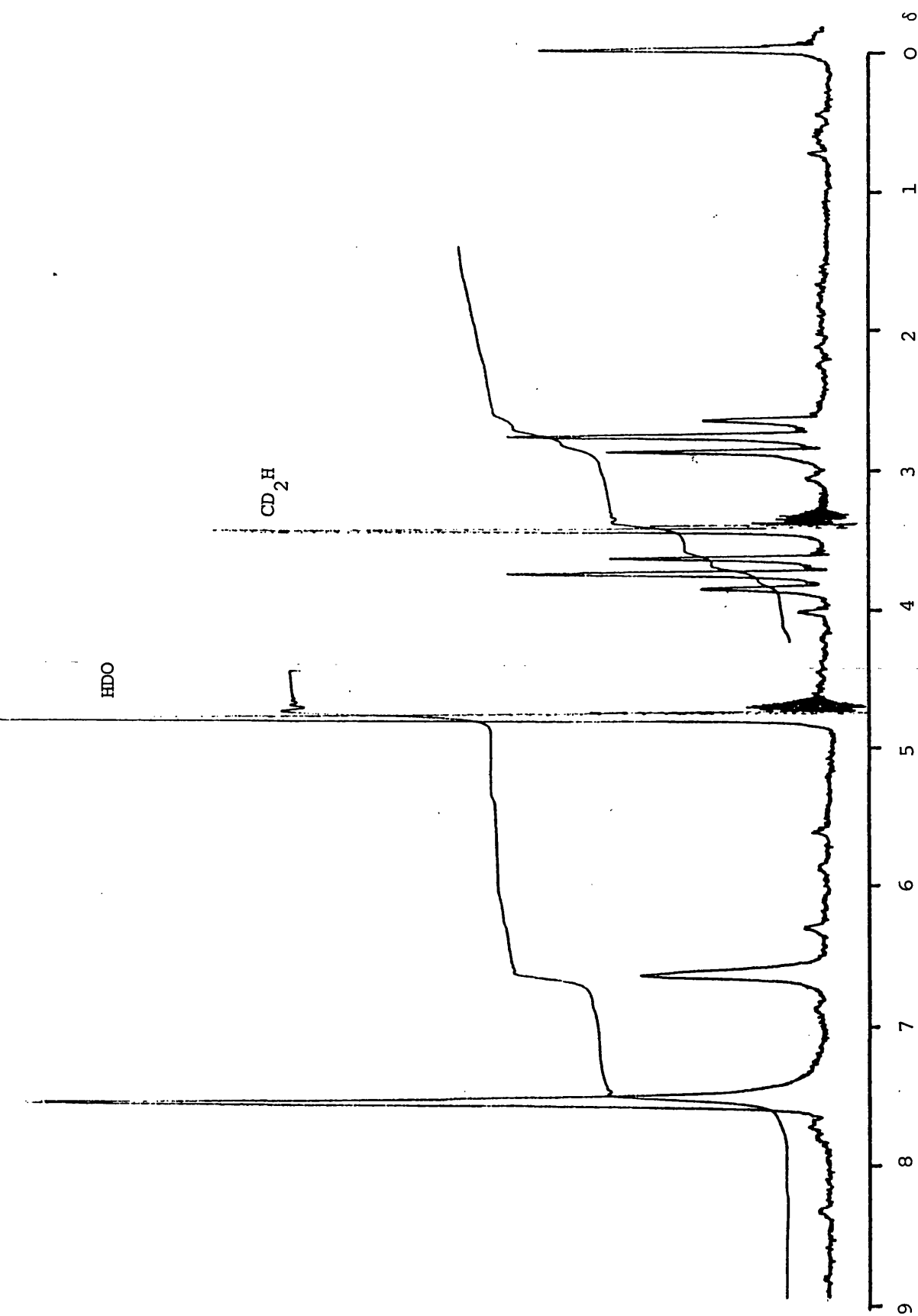


Figure 4.2. Proton (60 MHz) NMR spectrum of OPA/Mercaptoethanol in Methanol/Buffer (1:1).



of estimating the extent of reaction of gentamicin with OPA were sought. The feasibility of using fluorescence yield produced by the individual components as such a measure was investigated. Another spectroscopic method of assessing the extent of reaction was to use ninhydrin as a marker of unreacted amino groups. This presumed that ninhydrin would react with all of the primary amino groups on the gentamicins and fail to react with secondary functions.

4.2 Materials and Methods

4.2.1 Materials:

Ortho-phthalaldehyde (Sepramar), boric acid, mercaptoethanol, potassium hydroxide and Zerolit 325 (H^+) resin were all obtained from BDH Chemicals Ltd. Deuterium oxide, methanol- d_4 , acetonitrile- d_3 were supplied by the Aldrich Chemical Company Ltd. Methoxyethanol was obtained from Koch Light Laboratories Ltd.

4.2.2 Methods:

4.2.2.a. Nuclear Magnetic Resonance Study of the Reaction

Nuclear Magnetic Resonance

Proton 60 MHz spectra were determined on a JEOL PMX 60 SI spectrophotometer. Mr. H. Hartnell determined the carbon-13 NMR spectra on a JEOL FX 90Q Fourier Transform NMR spectrometer, in the School of Pharmacy and Pharmacology, University of Bath.

General Reagents and Solutions for NMR

Deuterium Oxide Buffer (pH 10.4)

Boric acid (1 g) was dissolved in distilled water (38 ml). The pH was adjusted to 10.4 with the use of a 45% w/v potassium hydroxide solution. Buffer solution (2 ml) was transferred to a flask and lyophilised. Deuterium oxide (2 ml) was used to redissolve the residue and the solution was again lyophilised. Deuterium oxide again was used to effect solution, which was stored in a desiccator.

Ortho-phthalaldehyde Reagent (OPA)

A general preparation used in the NMR studies was ortho-phthalaldehyde (35 mg) dissolved in methanol- d_4 (150 μ l) in a NMR tube. To this mercaptoethanol (20 mg) and deuterium oxide buffer (pH 10.4), 150 μ l, was added. Stability was adequate for several hours if protected from light.

Proton NMR Instrument Settings

Sweep width: 0 - 600 Hz

Sweep rate: 2.4 Hz/sec

Filter: 20 Hz

H_1 Level: 0.5

Phase: Pure Absorption Waveform

Amplitude: X 10

Internal Standards: sodium-3-trimethyl silyl propane
sulphonate (DSS) or tetramethyl-
silane (TMS)

Proton NMR Samples

Individual Reactants

Ortho-phthalaldehyde, mercaptoethanol and the amines used in the reaction were measured in various solvents depending on solubility. Amines used in the reaction apart from the gentamicin components were methylamine hydrochloride, butylamine, isopropylamine, glucosamine hydrochloride and ethanolamine.

OPA Reagent

Variations in the concentration and stoichiometry of the reaction mixture were studied. These included different ratios of OPA to mercaptoethanol and variation in the composition of solvents used.

OPA Reaction with Standard Amines

Amines were added to an already prepared OPA reaction mixture, either by weighing or by volume. Various proportions of amine to ortho-phthalaldehyde were used and spectra were run within ten minutes of initiation of reaction.

OPA Reaction with Gentamicin

Gentamicin C₁ (40 mg) was dissolved in deuterium oxide buffer (pH 10.4), 20 μ l and methanol-d₄ (150 μ l) in a NMR tube. OPA reaction mixture was prepared in a reaction vial by dissolving o-phthalaldehyde (45 mg) in methanol-d₄ (100 μ l)

and adding mercaptoethanol (46 μ l). The OPA reagent was transferred by microsyringe to the gentamicin solution and the vial was washed with methanol- d_4 (50 μ l) and added to the NMR tube. Spectra were run within ten minutes. Gentamicin C_2 was used in the same fashion except that 29 mg was used.

Carbon-13 NMR spectra

Samples of a similar description to those used in the PMR study were examined, except that acetonitrile was used instead of methanol as a co-solvent. Composition of each sample run is given:

- (i) OPA (35 mg) in acetonitrile (200 μ l)
- (ii) OPA (39 mg) and mercaptoethanol (45 mg) in acetonitrile (200 μ l)
- (iii) OPA (40 mg) and mercaptoethanol (23 mg) in deuteriated buffer (120 μ l) and acetonitrile (120 μ l)
- (iv) OPA (89 mg) and mercaptoethanol (52 mg) in deuteriated buffer (150 μ l) and acetonitrile (150 μ l)
- (v) OPA (30 mg) and mercaptoethanol (18 mg) in deuteriated buffer (150 μ l) and acetonitrile (150 μ l).
Ethanolamine (14 mg) was added to the mixture after solution of initial components was complete.

Internal reference in the spectra was set to acetonitrile methyl peak taken as 1.07 ppm in respect to TMS by reference to methanol (49.5 ppm). Proton NMR spectra of these solutions were recorded first then carbon-13 NMR spectra were run within one hour of preparation.

4.2.2.b Fluorescence Measurement of Gentamicin-OPA Reaction

A Perkin-Elmer 204A spectrofluorimeter was used to measure the extent of reaction. Excitation wavelength was set at 345 nm and emission at 440 nm with photomultiplier gain set at 2, and sensitivity of 1 used. Solutions of gentamicin components were prepared in concentrations of 5×10^{-7} M in 5 ml of ammonia-free methanol-water mixture (1:1). OPA reagent in the proportions of 4 molar and 3 molar equivalent with respect to each gentamicin component was added. The fluorescence obtained was measured 85, 110 and 120 minutes after the reaction started.

4.2.2.c Ninhydrin Reaction with Gentamicin

Preparation of Ninhydrin Reagent:

The Ninhydrin Reagent of the British Pharmacopeia 1980³⁰, was used in this determination. Preparation was as stated in Appendix 1A³⁰ with the double distilled water used having first been freshly boiled and cooled.

Preparation of OPA Reagent:

O-phthalaldehyde (50 mg) was dissolved in methanol (1 ml). To this mercaptoethanol (0.1 ml) was added and the solution made to 10 ml volume with 0.4 M borate buffer (pH 10.4). The methanol had been shaken with a Zerolit 325 (H⁺) resin and filtered prior to use. The buffer was freshly prepared using boiled and cooled distilled water.

Preparation of 95% Ammonia-free Ethanol-Water (1:1):

Ethanol, 95% v/v, 50 ml was shaken with a Zerolit 325 (H^+) resin for five minutes, then allowed to stand for an hour with intermittent shaking. Water (50 ml) which had been double distilled was boiled and allowed to cool before adding to the filtered ethanol. All solutions were freshly prepared each day.

Gentamicin Standard Solutions:

Standard solutions for each of the three gentamicin components (C_{1a} , C_2 and C_1) were prepared by dissolving base (20 mg) in water (10 ml) to give 2 mg/ml solutions.

Calibration of Ninhydrin Reaction with Gentamicin:

Five aliquots of gentamicin component standard solution (15, 30, 45, 60 and 75 μ l) were transferred to separate test tubes and the volumes made up to 1 ml with water. Ninhydrin reagent (1 ml) was added; the solutions mixed on a vortex mixer and placed in a boiling water bath for twenty minutes. At the end of this time the solution was allowed to cool for five minutes then 5 ml of the 95% ammonia-free ethanol-water mixture was added. The absorbance was immediately measured on a Unicam SP 600 spectrophotometer at 565 nm using 1 cm matched cells against a blank prepared without the gentamicin present. This was repeated for all three components and a five point calibration curve was constructed for each.

Ninhydrin reaction with Gentamicin in Presence of

OPA Reagent:

To four test tubes containing 75 μ l of the gentamicin component solutions were added varying amounts of OPA reagent.

For gentamicin C_{1a} volumes of 7, 15, 22 and 30 μ l OPA added.

gentamicin C₂ volumes of 7, 14, 21 and 29 μ l OPA added

gentamicin C₁ volumes of 5, 10, 16 and 21 μ l OPA added.

After addition of the OPA reagent, water was added to give 1 ml of solution; this was vortex mixed and kept in the dark for fifteen minutes. At the end of this period, 1 ml of ninhydrin reagent was added, the solution mixed and put in the boiling water bath for twenty minutes. Throughout this period the solution was protected from light. After cooling for five minutes, the 95% ammonia-free ethanol-water mixture (5 ml) was added. Absorbance was read at 565 nm against a blank.

A further five test tubes of the gentamicin components (75 μ l) had the following amounts of OPA reagent added.

gentamicin C_{1a} 11, 22, 30, 34 and 37 μ l

gentamicin C₂ 11, 21, 29, 32 and 36 μ l

gentamicin C₁ 8, 16, 21, 23 and 26 μ l

These were reacted as previously and the absorbances read at 565 nm.

4.3 Results and Discussion

4.3.1 Nuclear Magnetic Resonance Study of the Reaction

Mechanism between o-Phthalaldehyde and Primary Amines

4.3.1.a NMR of Individual Reactants:

The proton NMR of o-phthalaldehyde is shown in Figure 4.1 and is in accord with published data²⁹⁷. The proton spectrum of mercaptoethanol is illustrated in Figure 4.3. The singlet at $\delta 4.68$ is the hydroxy proton whilst the neighbouring methylene group has a triplet at $\delta 3.7$ with a coupling constant (J) of about 7 Hz. The other methylene group exhibits a signal at $\delta 2.7$ of two overlapping triplets. The thiol proton is seen as a multiplet at $\delta 1.8$. In deuterium oxide solutions the thiol and hydroxyl protons are not observed, and the methylene groups appear as triplets at the chemical shifts indicated.

Ethanolamine, in acetonitrile/aqueous buffer, is similarly seen as two triplets at $\delta 3.6$ and $\delta 2.7$ ($J \approx 7$ Hz), with the hydroxy bearing methylene group giving the lowest field signal. The spectrum is shown in Figure 4.4.

Glucosamine gives a proton NMR spectrum as shown in Figure 4.5, with a complicated splitting and overlapping of signals from $\delta 2.8$ to 4.8. The anomeric proton is observed as a doublet ($J = 4$ Hz) at $\delta 5.5$.

Gentamicin C₂, in deuterium oxide, exhibits a spectrum (Figure 4.6) similar to glucosamine. The large number of protons in a similar environment defy identification. However the two

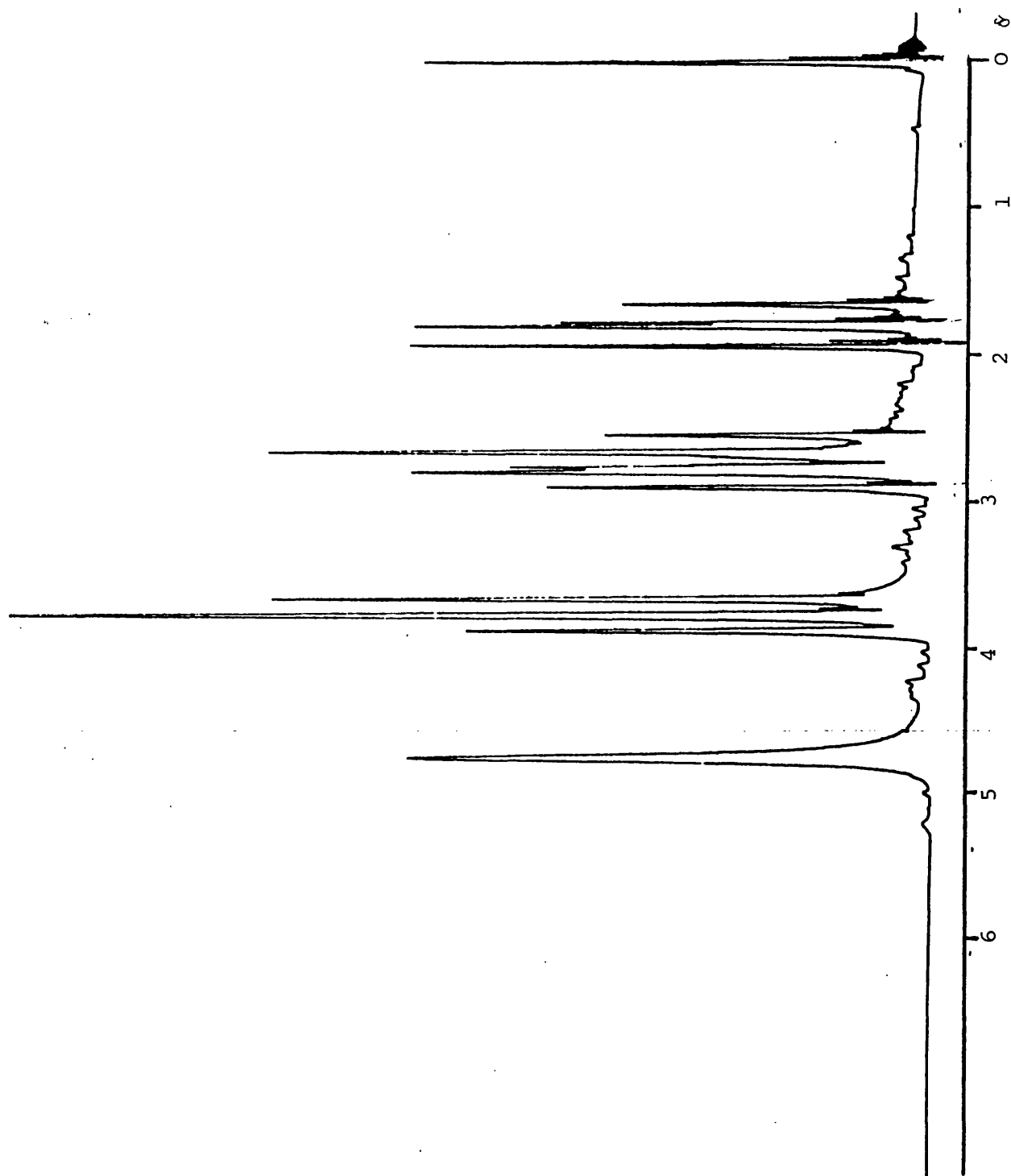


Figure 4.3. Proton (60 MHz) NMR spectrum of Mercaptoethanol

Figure 4.4. Proton (60 MHz) NMR spectrum of Ethanolamine in Acetonitrile/Buffer.

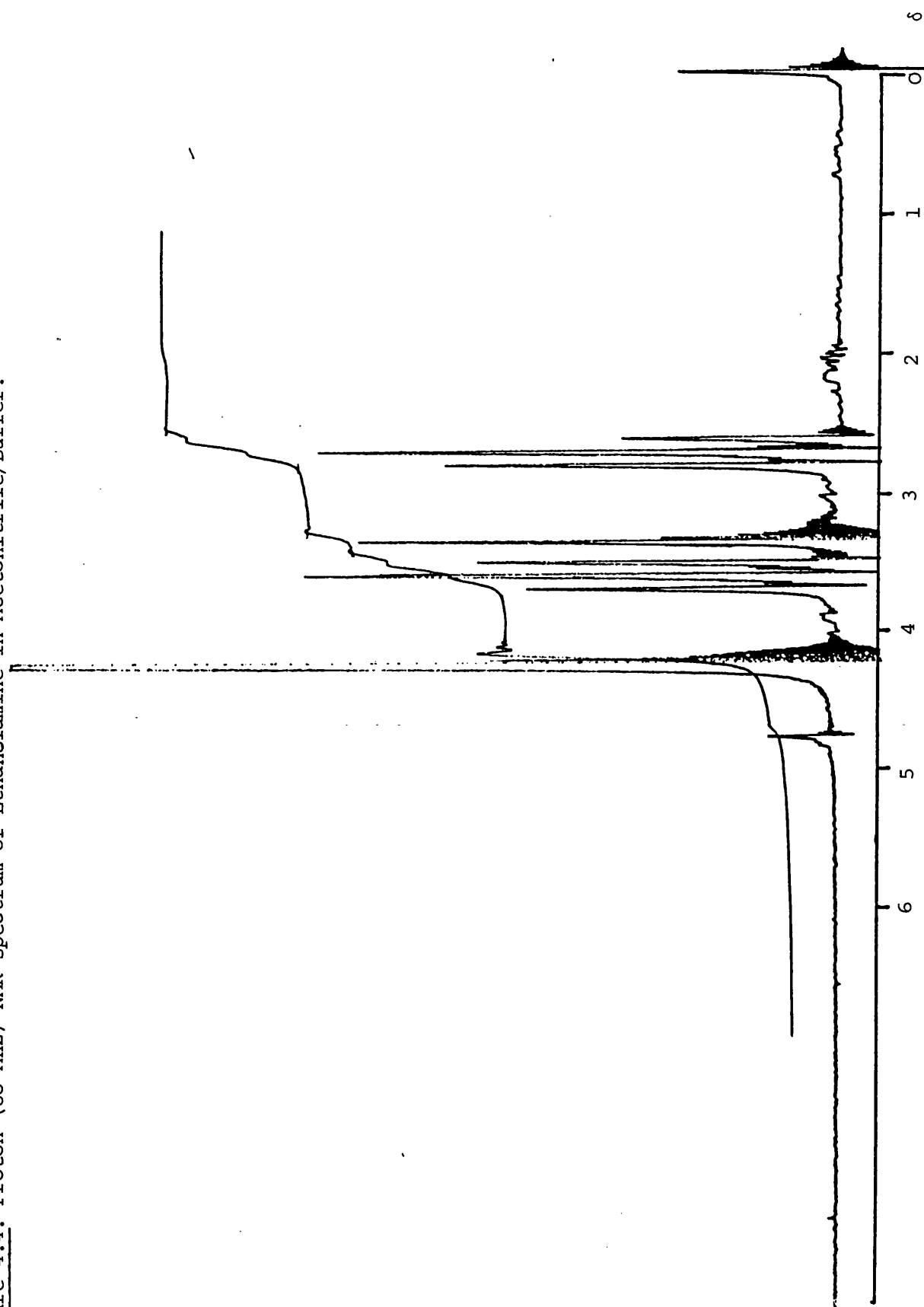


Figure 4.5. Proton (60 MHz) NMR spectrum of
Glucosamine in Deuterium Oxide.

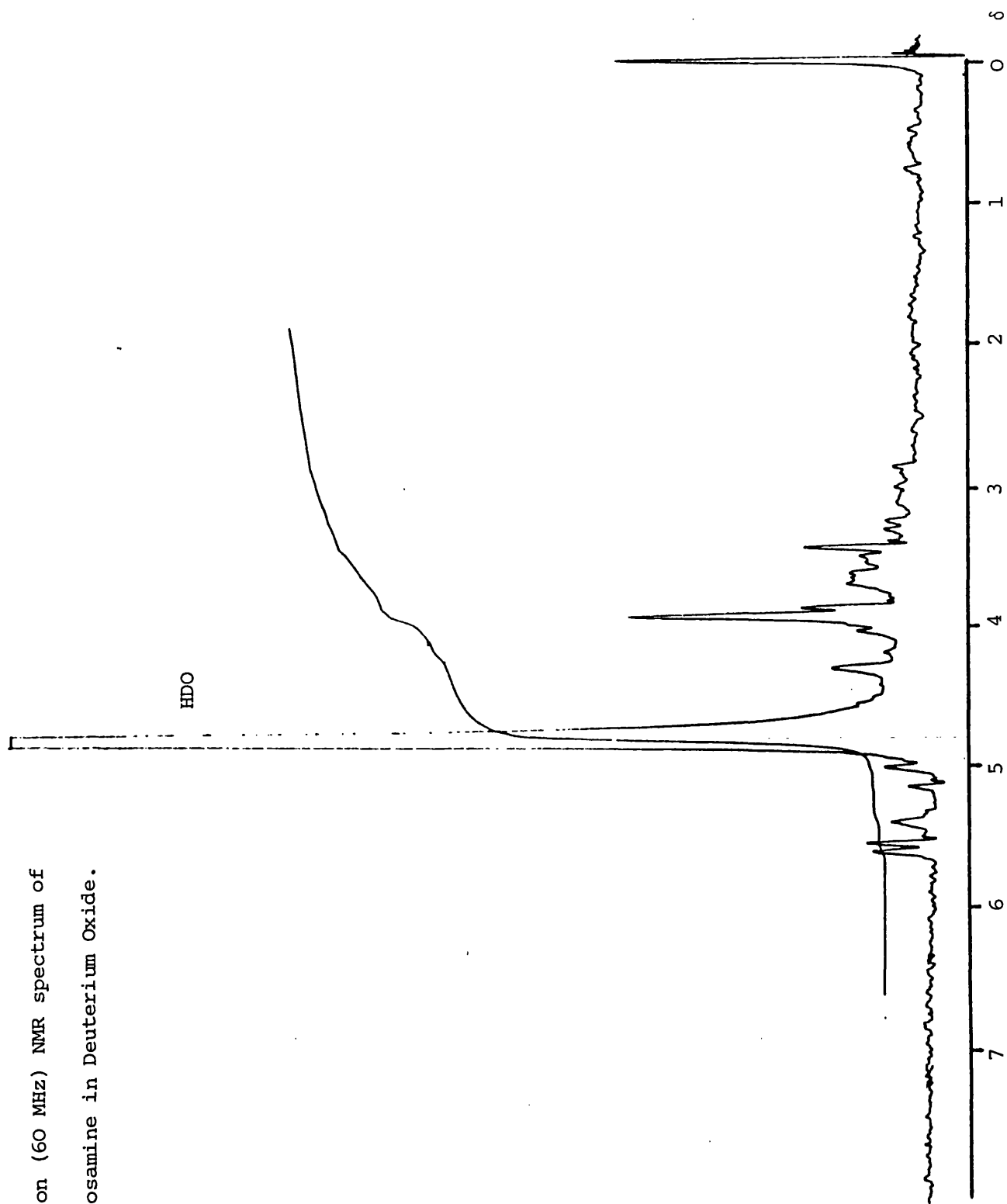
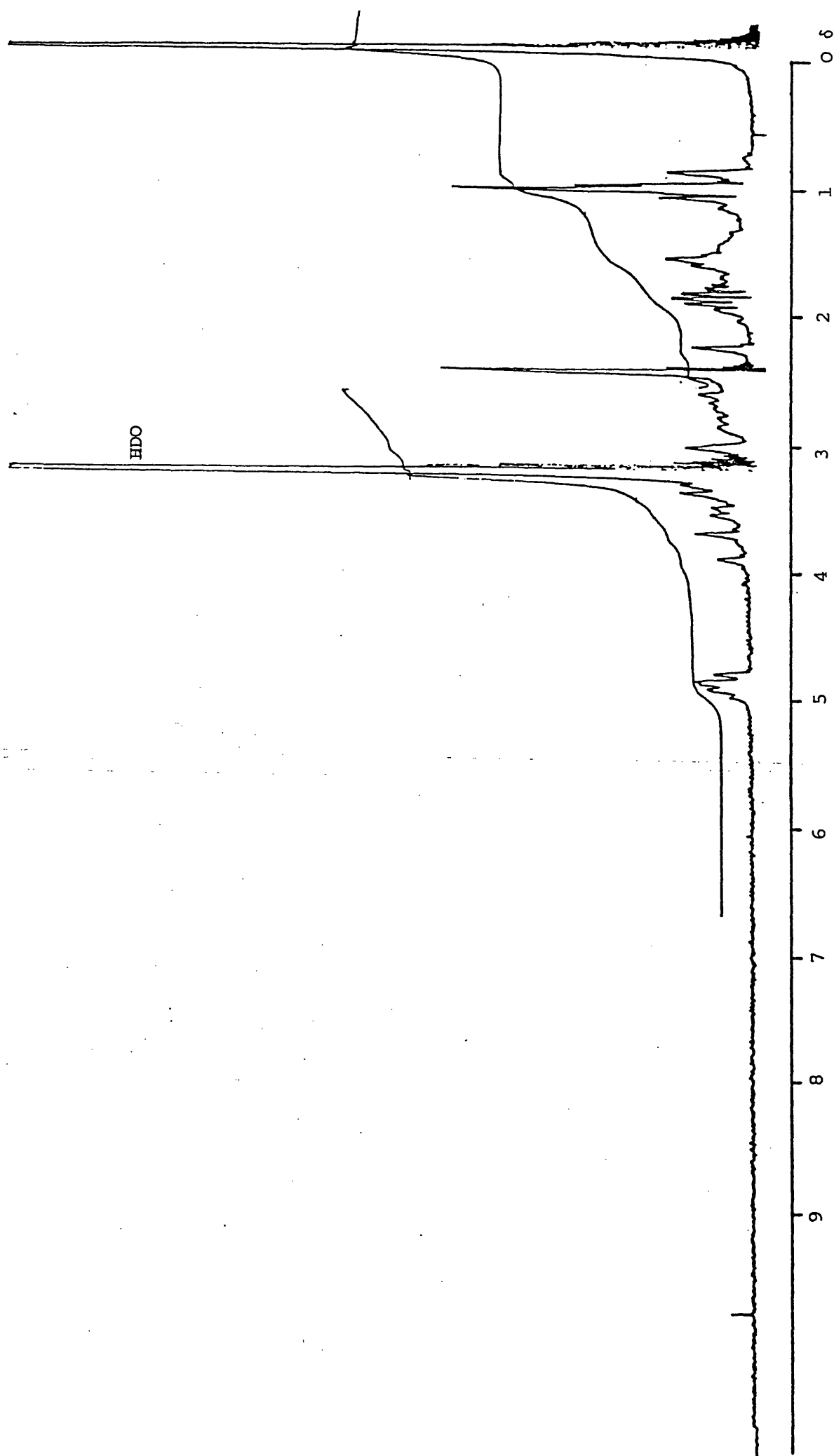


Figure 4.6. Proton (60 MHz) NMR spectrum of Gentamicin C₂ in Deuterium Oxide.



anomeric protons at $\delta 4.9 - 5.2$ are readily seen, as are the singlets due to methyl groups at $\delta 2.58$ and $\delta 1.14$.

4.3.1.b NMR of o-Phthalaldehyde Reagent:

The composition of the OPA, in the reagent conditions used for pre-column derivatisation, was studied. The OPA reagent consists of OPA and mercaptoethanol dissolved in an alkaline buffer solution with a small amount of methanol also present. Under reaction conditions the level of methanol is greater, and approaches 50% v/v. Methanol was not always used in the NMR experiment because of poor spectra and solubility difficulties. Acetonitrile was used instead as co-solvent, because of its polar characteristics and the better solubility of some of the compounds used in the study.

To assess the changes in the OPA in the reaction mixture the proton and carbon-13 NMR spectra of the dicarbonyl compound (4.III) was taken as standard. The proton spectrum of OPA in chloroform is given in Figure 4.1, whilst the carbon-13 spectrum is shown in Figure 4.7 of OPA in acetonitrile. The resonances obtained from Figure 4.7 are assigned in Table 4.1. In this table, a comparison with literature values²⁷⁴ for OPA is made, with the different solvent perhaps contributing to the slight difference in chemical shifts. It is readily apparent from Figures 4.1 and 4.7 that the aldehyde groups are clearly denoted in both types of NMR spectrum.

Figure 4.7. Carbon-13 NMR spectrum of o-phthalaldehyde in Acetonitrile.

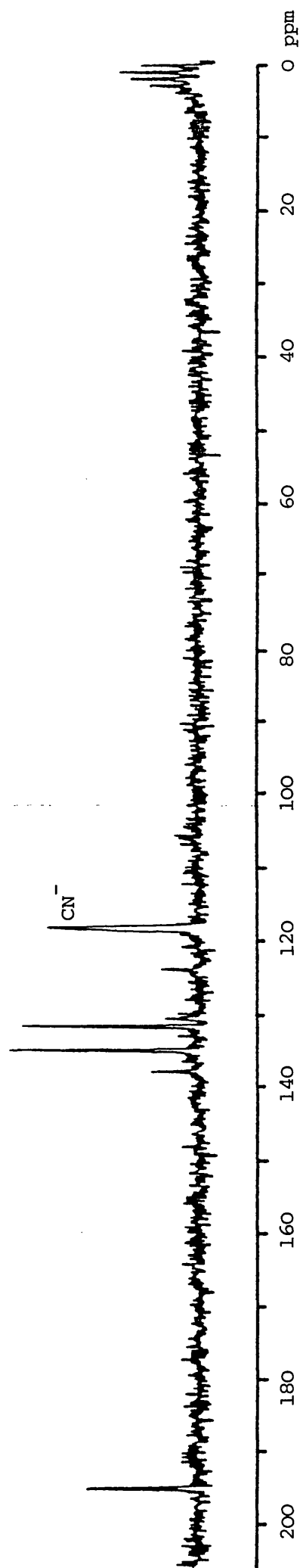
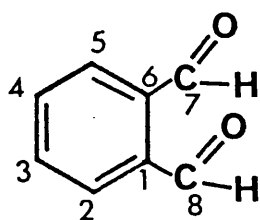


Table 4.1. Carbon-13 NMR of OPA in acetonitrile



(4.III)

Carbon	Off. Res.	Chemical Shift (ppm)	Literature (CFCl ₃ ;ppm)
7,8	d	193.61	196.10
1,6	s	137.32	137.20
3,4	d	134.50	135.80
2,5	d	131.25	133.10

When methanol was added to the chloroform solution of OPA (Figure 4.8) a large singlet appeared at $\delta 7.48$ in the proton spectrum. A series of small peaks in the $\delta 6 - 6.8$ region were also visible. The integral of the aldehyde protons was reduced with respect to that of the aromatic protons, presumably due to a nucleophilic attack on the carbonyl by the methanol, forming a hemiacetal function.

OPA in acetonitrile gives a similar spectrum to OPA in chloroform, but on the addition of deuterium oxide buffer in the proportion of 1:1 (Figure 4.9), two singlets appear at $\delta 7.56$ and $\delta 6.55$ which appear to correspond to those found in Figure 4.2. The strong peak at $\delta 7.56$ was assumed to be the

Figure 4.8. Proton (60 MHz) NMR spectrum of OPA in

Chloroform and Methanol.

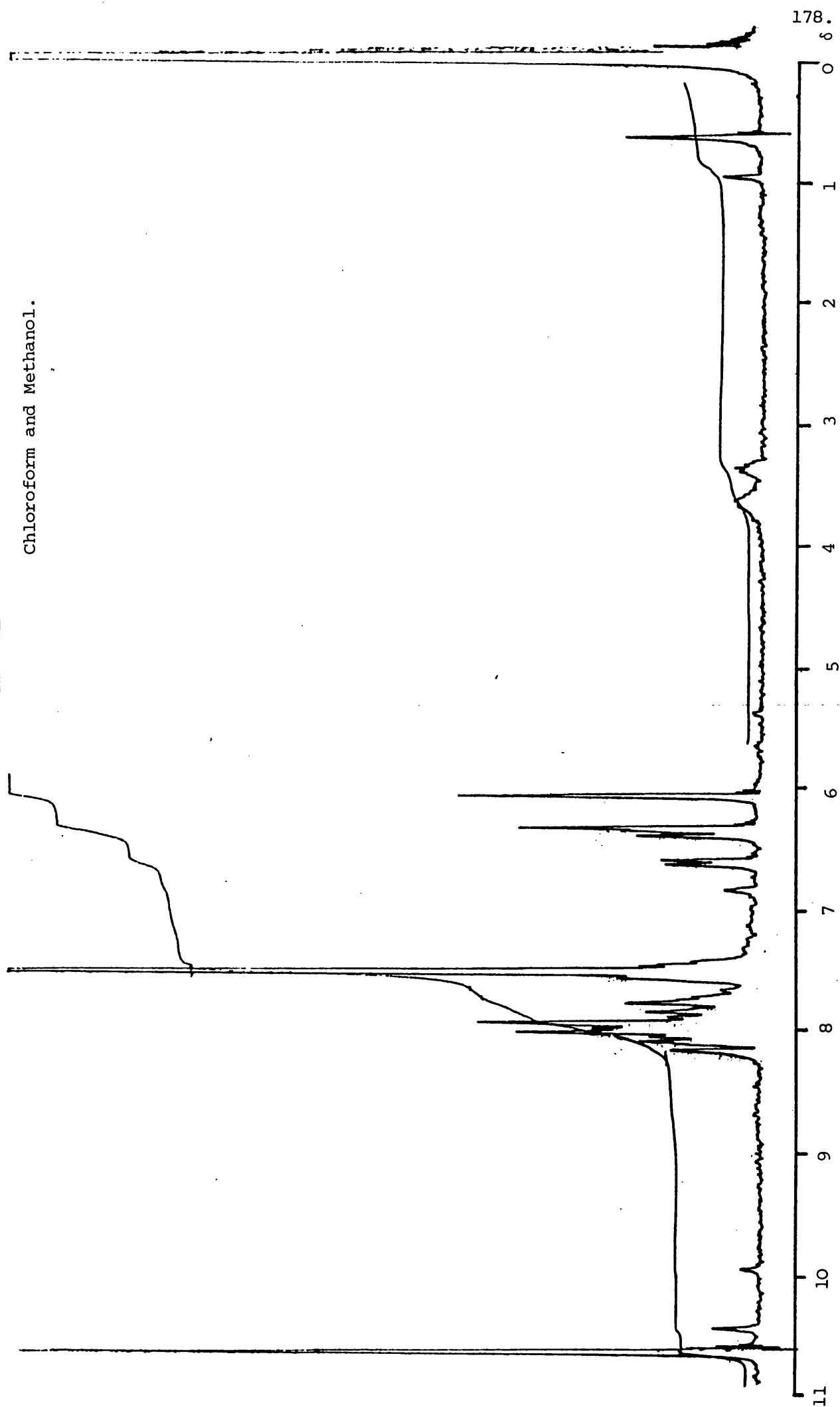
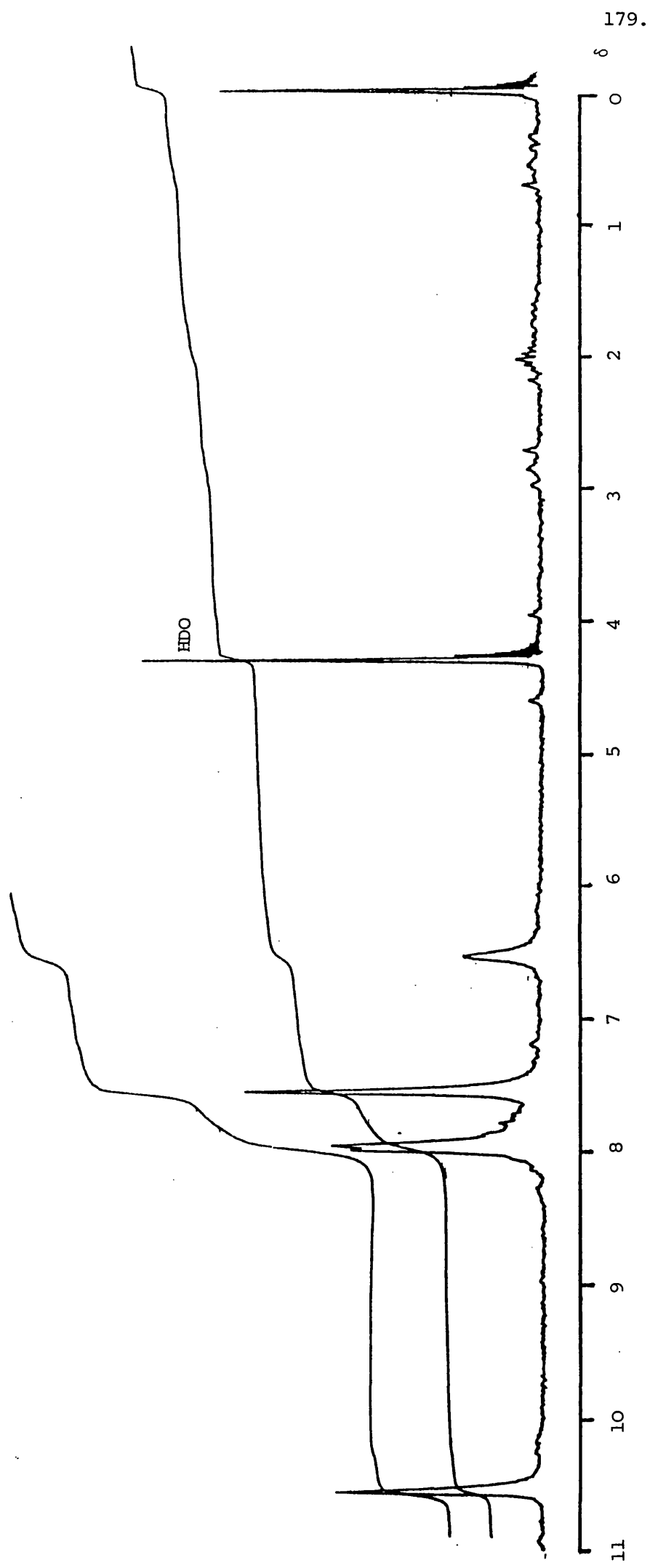


Figure 4.9. Proton (60 MHz) NMR spectrum of OPA in Acetonitrile/Buffer.



aromatic protons of a new species of compound present. The singlet at 6.55 could represent a nucleophilic addition of hydroxy ions from the base to one or both of the carbonyl functions.

On addition of mercaptoethanol to OPA dissolved in acetonitrile a four proton singlet of aromatic protons is again formed (Figure 4.10). However, this time a complex splitting of signals occurs in the $\delta 6-7$ region. The thiol promoted the production of the four proton aromatic singlet at $\delta 7.5$ and a linear relationship between the integral ratio of "old" to "new" aromatic signal, and concentration of mercaptoethanol was seen (Figure 4.11). The correlation coefficient of the four point plot was 0.9996, with a slope of 1.90 and intercept of -0.60. The "new species" is present in a proportion of over 50% at equal concentrations of mercaptoethanol and OPA, and 75% when mercaptoethanol is in a two fold excess of OPA.

Addition of deuterium oxide made no significant difference to the spectrum of such a mixture containing mercaptoethanol. However, the addition of deuterium oxide buffer (pH 10.4) to the solution removed all traces of the aldehyde singlet, the aromatic multiplet and the various split peaks in the $\delta 6.4 - 6.6$ region. The proton NMR spectrum of equal concentrations of OPA and mercaptoethanol in acetonitrile/deuterium oxide buffer (1:1) is given in Figure 4.12. This can be directly compared to Figure 4.2 which had exactly the same

Figure 4.10. Proton (60 MHz) NMR spectrum of OPA in Acetonitrile and Mercaptoethanol.

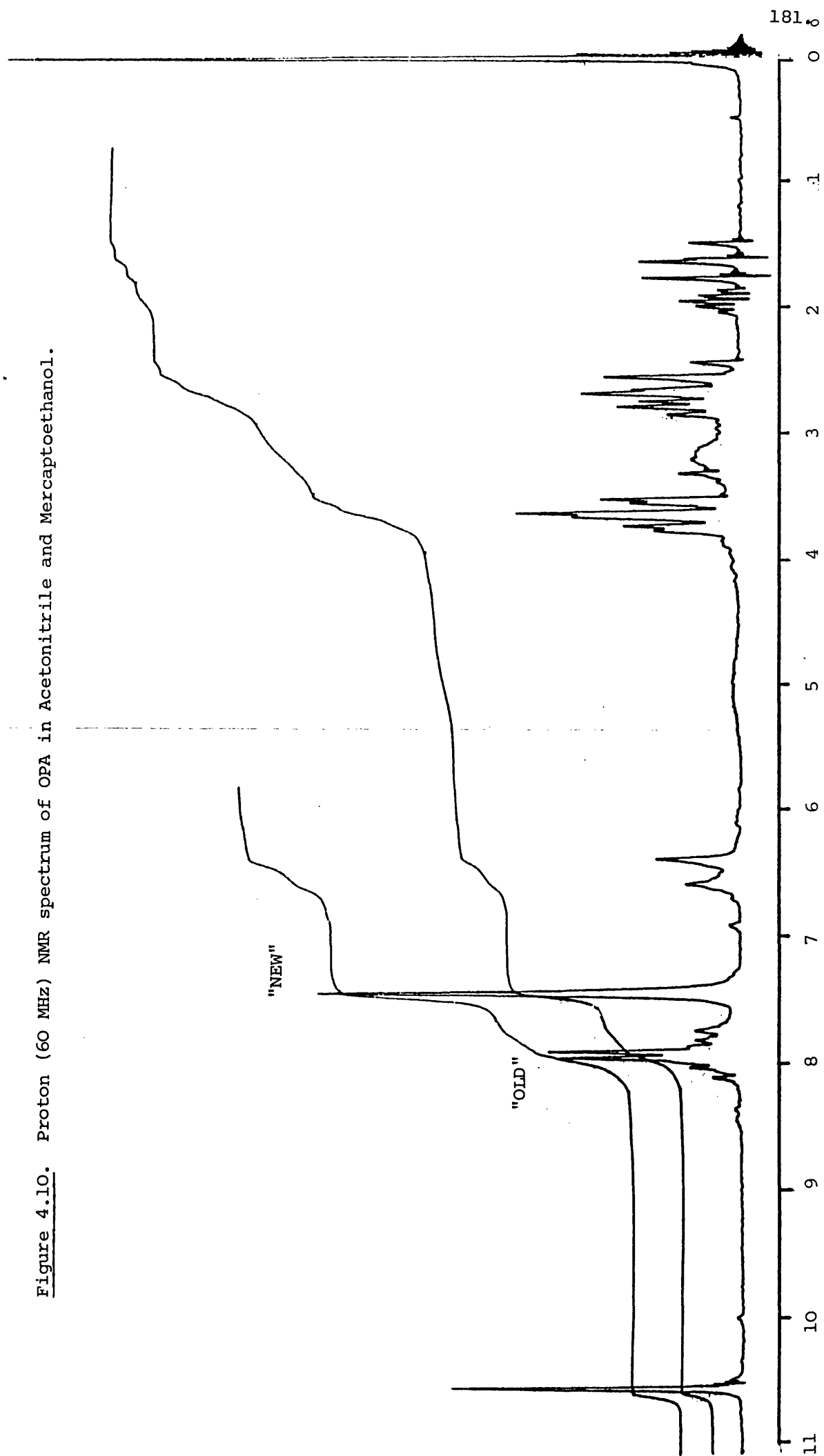


Figure 4.11. Relationship of appearance of 'new' Aromatic Singlet ($\delta 7.5$) to Mercaptoethanol concentration.

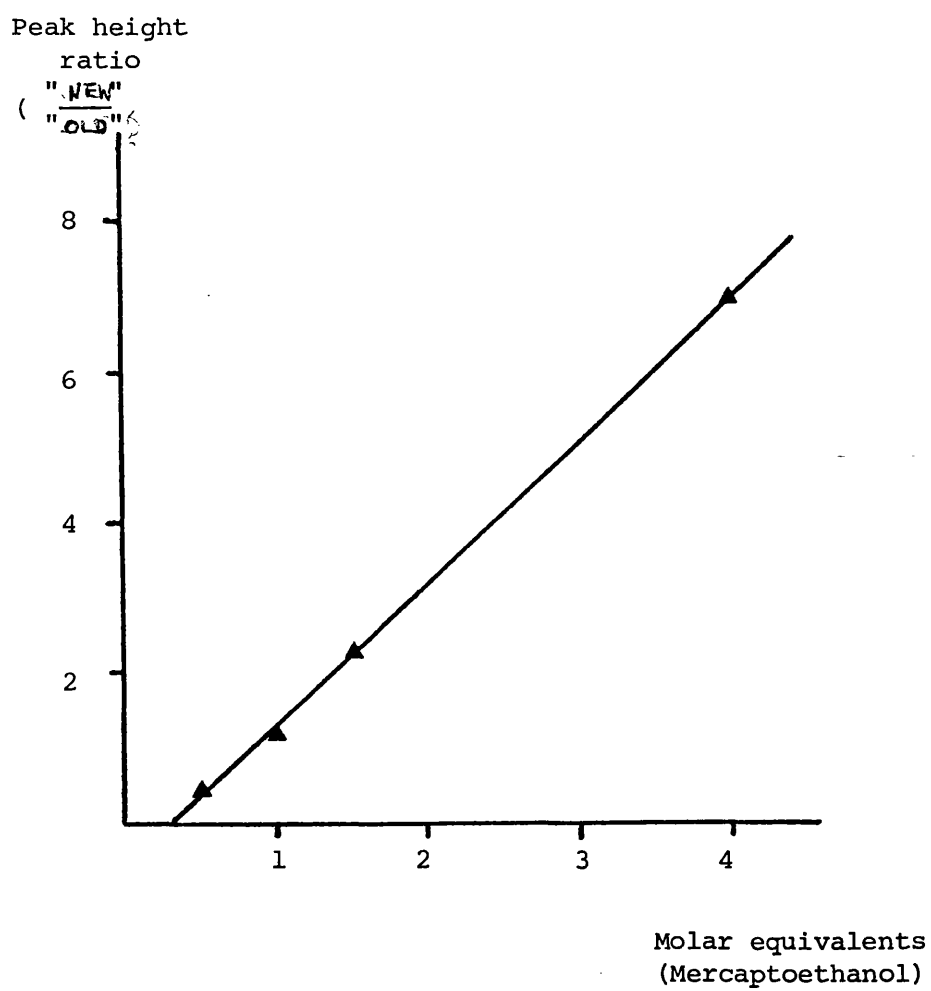
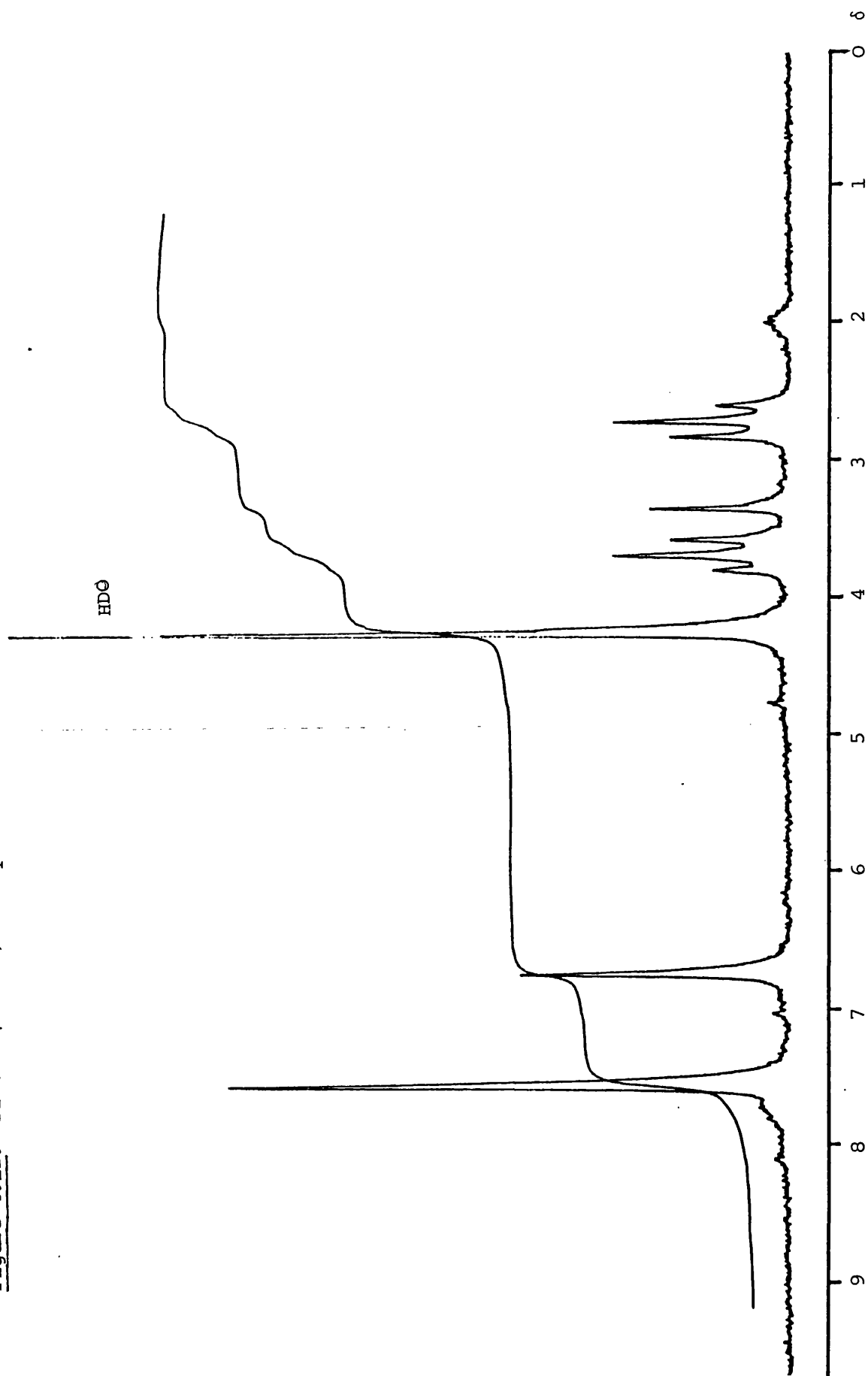


Figure 4.12. Proton (60 MHz) NMR Spectrum of OPA in Acetonitrile/Buffer



composition, except that methanol replaced acetonitrile as the co-solvent. Both spectra reveal a four proton aromatic singlet at $\delta 7.5$ and a two proton singlet at $\delta 6.7$. The carbon-13 NMR spectrum of the reagent mixture in acetonitrile/buffer solution is shown in Figure 4.13. Chemical shifts with possible assignments are given in Table 4.2. The shifts for the 7 and 8 carbons are approximate and correspond to two broad disturbances of the baseline in Figure 4.13. It is assumed that there must be an equilibrium in the solution, possibly as indicated by the two species (4.IV) and (4.V). There are no aldehyde peaks visible in Figure 4.12 or 4.13 and this may suggest the total absence of (4.IV) due to the reactive environment. The hydroxy ions in the buffer are probably

Table 4.2. Carbon-13 NMR Chemical Shifts of Figure 4.13.

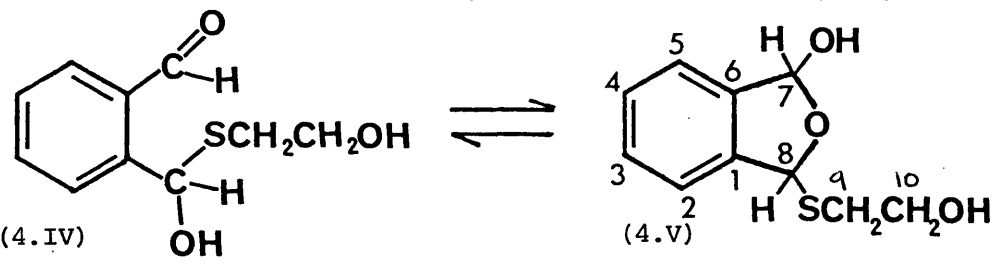
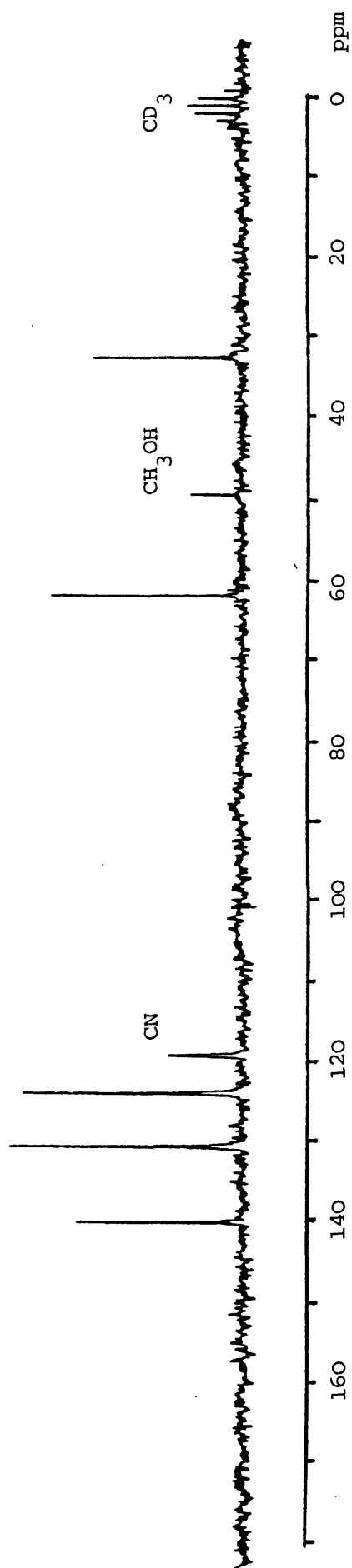
		
Carbon	Off. Res.	Chemical Shift (ppm)
1,6	s	139.43
4,3	d	130.22
2,5	d	123.66
9	t	32.45
10	t	61.96
7	-	~103
8	-	~88

Figure 4.13. Carbon-13 NMR Spectrum of OPA in Acetonitrile/Buffer.



responsible for a higher activation of the thiol as a nucleophilic species by removal of the acidic proton, but they may also destabilise the formed product (4.V). The carbonyl species represented by (4.IV) may be present with a diol function on the C-7 instead of the aldehyde. The broad singlet at $\delta 6.7$ in the proton spectrum and the absence of sharp signals for the C-7 and C-8 atoms in the carbon-13 spectrum, both indicate that the reaction mixture does not contain a single species, but at least two very reactive intermediates.

To further characterise the mixture, a more concentrated solution of OPA and mercaptoethanol in the acetonitrile/buffer solvent was made. The proton NMR spectrum is given in Figure 4.14 and carbon-13 NMR in Figure 4.15 with resonances assigned in Table 4.3. The proton spectrum shows the singlet at $\delta 6.7$ to be split into three broad overlapping peaks. There is also a broadening of the thiol methylene group, probably due to splitting, and a very small aldehyde peak is also present. In the carbon-13 spectrum in Figure 4.15 the signals of the C-7 and C-8 atoms are readily apparent at similar chemical shifts to those given in Table 4.2, which confirms the assignment of Figure 4.13. A splitting of the signals of the aromatic carbons may be due to the individual carbons being in slightly different environments in respect to the hydroxy and thioether functions. Or it may be due to different isomeric compounds present in the mixture, occurring because of the asymmetric centres at C-7 and C-8. The C-9 atom of the thio substituent is deshielded by 4 ppm and the C-10 is shielded by 3 ppm in respect to the free mercapto-

Figure 4.14. Proton (60 MHz) NMR Spectrum of OPA Reaction Mixture (concentrated) in Acetonitrile/Buffer.

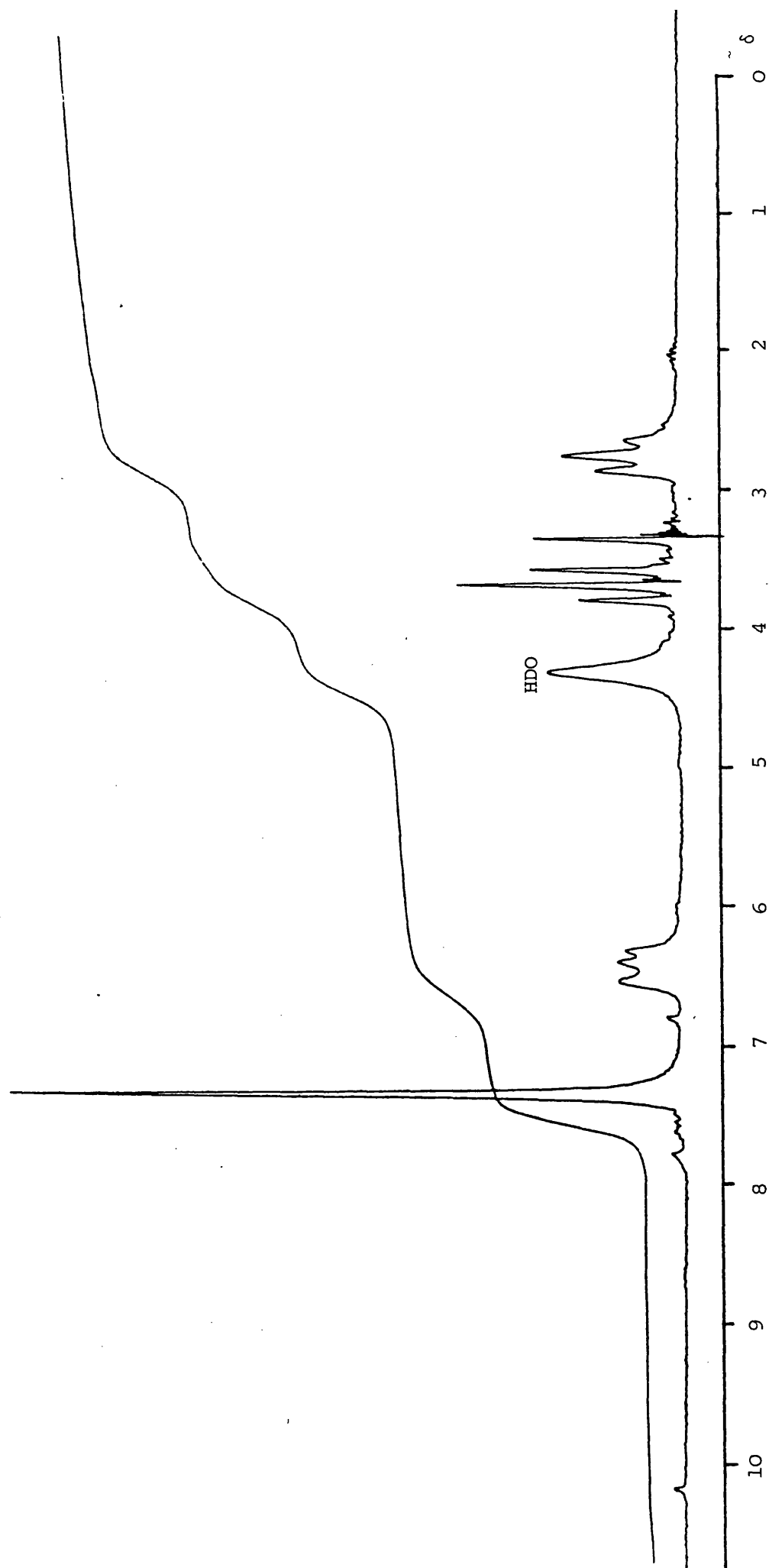


Figure 4.15. Carbon-13 NMR Spectrum of OPA (concentrated Reagent) in Acetonitrile/Buffer.

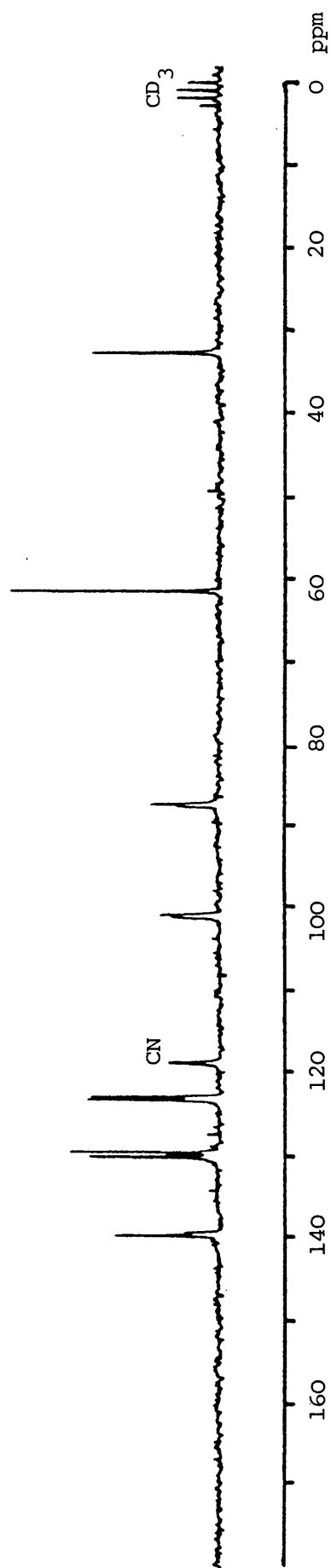
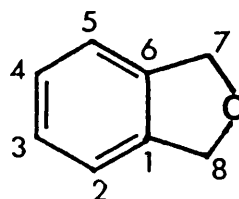
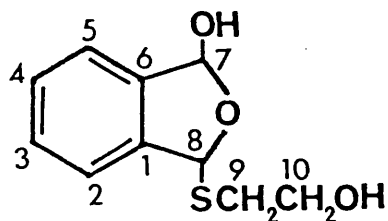


Table 4.3. Chemical shifts of carbon-13 NMR spectrum of

Figure 4.15 with dihydroisobenzofuran compared



(4.VI)

Carbon	Off. Res.	Chemical shift (ppm)	Chemical shift (4.VI; ppm)
1,6	s	138.62/138.24	139.40
4,3	d	129.30/128.71	127.20
2,5	d	122.48/122.10	121.00
7	d	100.37/100.07	73.50
8	d	86.67	
9	t	31.79	
10	t	60.82	

ethanol. This is consistent with the mercaptoethanol being adjacent to an aryl and ether function. Overall the resonances given in Table 4.3 are very similar to those in Table 4.2 indicating the presence of a common intermediate in both. A comparison of these shifts to those for 1,3 dihydro-isobenzofuran²⁷⁴ reveal a very similar pattern. It is likely that the intermediate present in the concentrated solution is a dihydro-isobenzofuran derivative with a more stable structure as determined

by NMR, due to an effectively lower concentration of hydroxide ions compared to the concentration of the reagent species. McDonald³⁵⁰ found that 90% of OPA in aqueous solution existed as the 1,3-phthalandiol (1,3-dihydroxy,1,3 dihydro-isobenzofuran) and this has been observed by other workers³⁵¹. Simons²⁸⁹ discovered the 1,3-disubstituted, 1,3-dihydroisobenzofuran (4.V) intermediate to be present in the OPA/mercaptoethanol mixture in ethanol, and found it to be in equilibrium with the starting materials. He described the proton spectrum as having an aromatic signal at $\delta 7.33$ and the C 7,8 protons as two pairs of unequal singlets centred at $\delta 6.45$. However, he considered this compound to be unimportant in the reaction with amines.

Since the reaction mixture used in the HPLC analysis has a four-fold molar excess of mercaptoethanol compared to OPA, the effect of increasing the concentration of mercaptoethanol over the 1:1 ratio used previously was investigated. An excess of mercaptoethanol in an acetonitrile or methanol/deuterium buffer mixture only sharpened the proton spectrum of Figures 4.2 or 4.12. A two-fold excess of mercaptoethanol to OPA in pure acetonitrile gave the proton spectrum illustrated in Figure 4.16, and the carbon-13 NMR spectrum in Figure 4.17, with shift values assigned in Table 4.4. The proton spectrum showed aldehyde peaks soon after the reaction mixture was made up. The signal decreased with time, and when the carbon-13 spectrum was produced there was little sign of the aldehydes. The proton spectrum shows the two oddly split singlets which must be due to the C-7 and C-8

Figure 4.16. Proton (60 MHz) NMR Spectrum of OPA Reaction Mixture in Acetonitrile with a Twofold

excess of Mercaptoethanol.

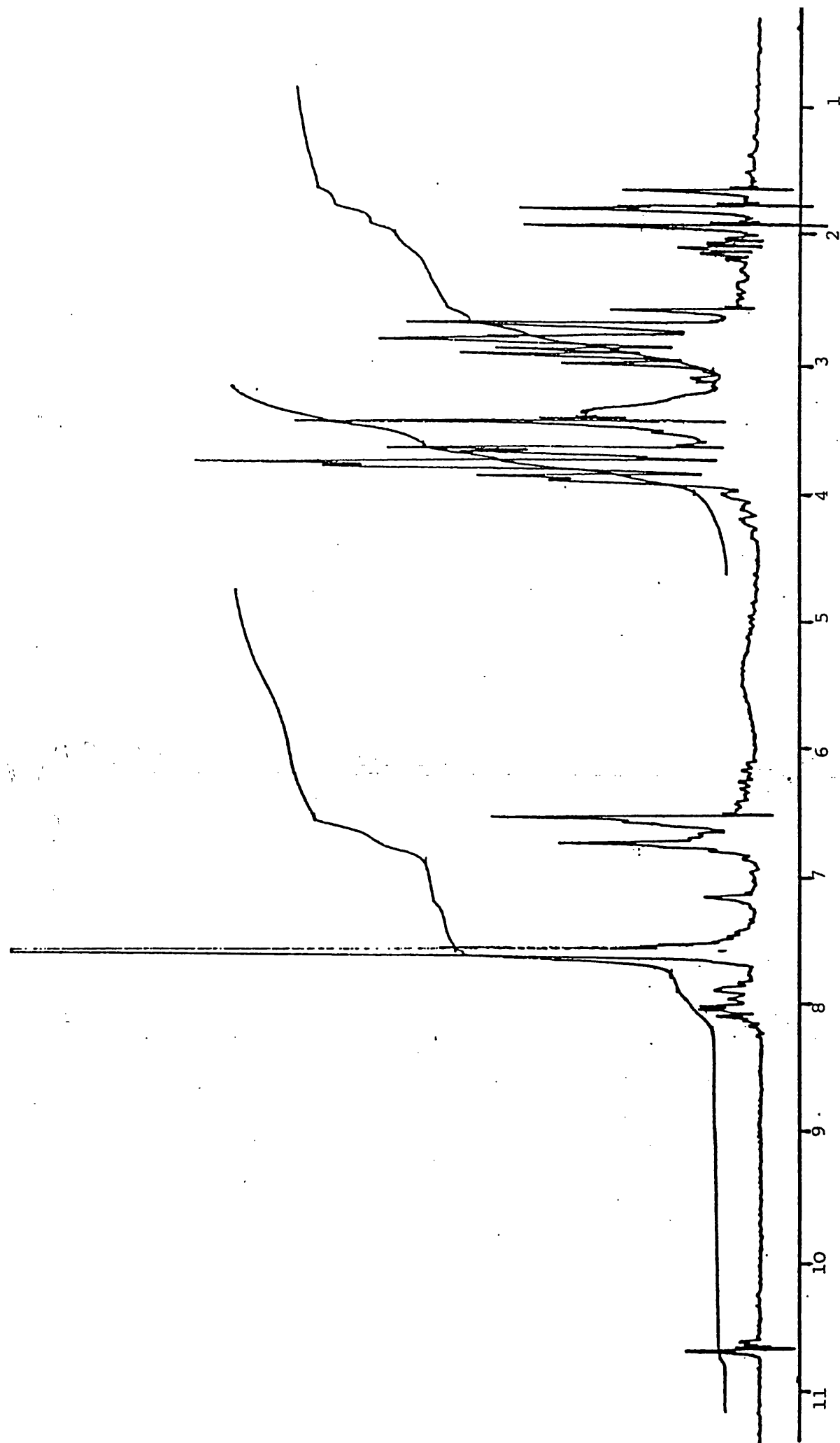


Figure 4.17. Carbon-13 NMR Spectrum of OPA in Acetonitrile with a Twofold Excess of Mercaptoethanol.

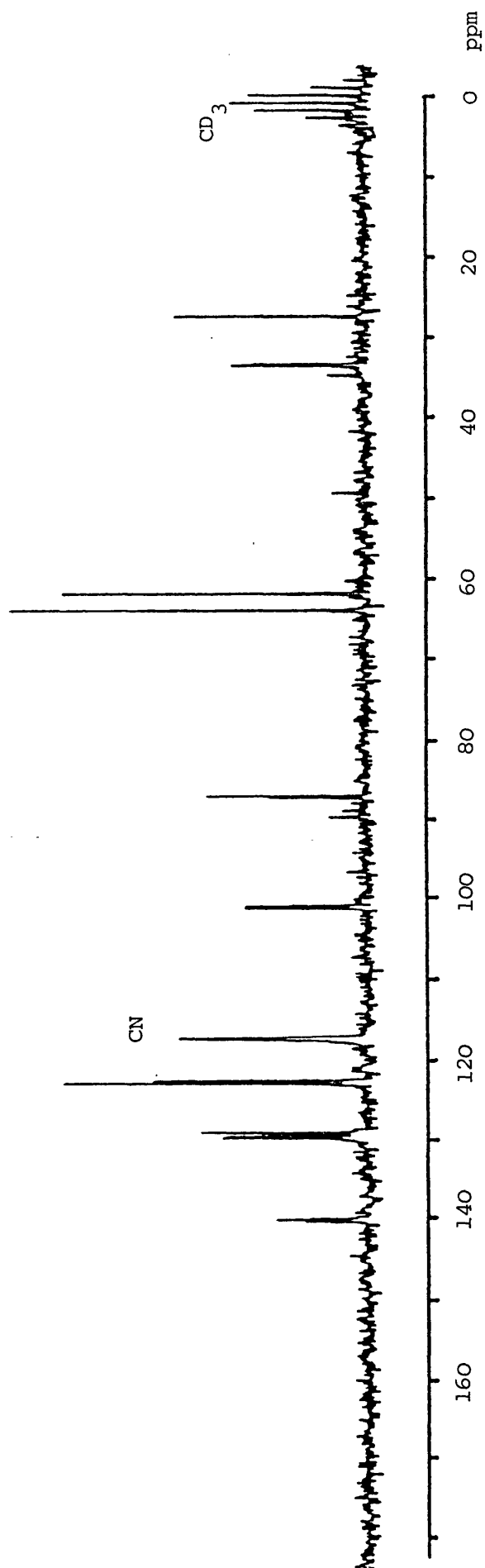
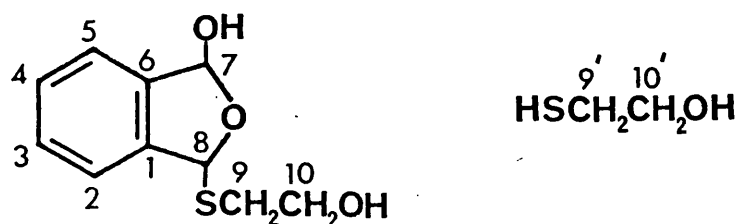


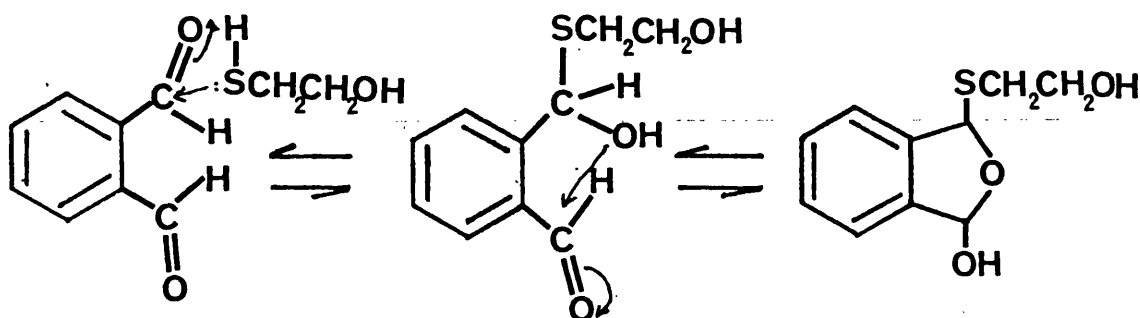
Table 4.4. Carbon-13 chemical shifts of OPA with two-fold excess of mercaptoethanol in acetonitrile.



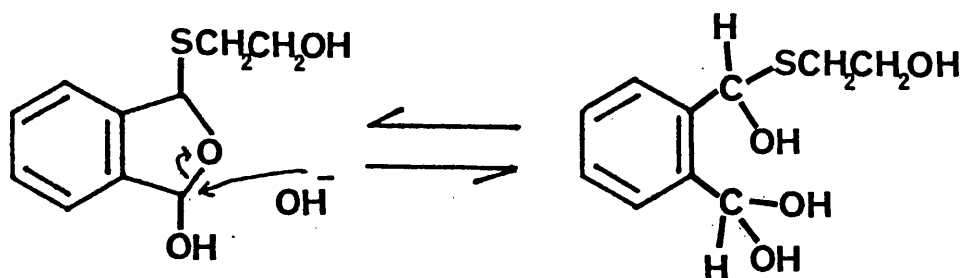
Carbon	Off. Res.	Chemical Shift (ppm)
1,6	s	140.40/140.19/140.07
3,4	d	130.16/130.06/129.57/ 129.46
2,5	d	123.40/123.18
7	d	101.67/101.40
8	d	87.80/87.64
9	t	34.76/35.52/33.35
10	t	62.28
9'	t	27.44
10'	t	64.40

protons. The thiol proton is also present as signified by the triplet at $\delta 1.8$. This is further confirmed by the difference in shifts of the two sets of triplets for the methylene group adjacent to the thiol function ($\Delta \delta 0.1$). Thus from Figure 4.16 it appears that only about 40% of the mercaptoethanol has reacted as quantified by integrals. The carbon-13 NMR spectrum confirms only a partial reaction, with the excess mercaptoethanol chemical shifts denoted by 9' and 10'.

This figure also shows more splitting of the aromatic carbons than in Figure 4.15, which could indicate the formation of more stable compounds than on the acetonitrile/buffer mixture. If there is an equilibrium present it is very slow and thus all possible conformations of (4.V) can be registered. The equivalence of the carbon-13 NMR spectra of these three mixtures (Figures 4.13, 15 and 17), indicate that the reactive intermediate is the same for each, with the only difference being the relative stability of the compound. The excess of thiol clearly did not produce significant amounts of a disubstituted intermediate. The reaction mechanism in this medium may be as shown by:



In acetonitrile there is presumably little reverse reaction, the aldehyde peaks being produced by unreacted OPA. In methanol the structure would be more unstable due to nucleophilic attack by the solvent at the hemiacetal carbon. The presence of hydroxy ions would exert a similar influence although the formation of more than transient carbonyl bonds appears to be highly unlikely. The reaction mixture used in the HPLC assay is thus considered to be a rapid equilibrium, possibly of the type shown:



4.3.1.c o-Phthalaldehyde Reaction with Standard Amines

The OPA reaction, using equimolar concentrations of OPA and mercaptoethanol in methanol/buffer, with methylamine as amine substituent gave a black precipitate in a dark brown solution. This may have been a decomposition product since the proton NMR spectrum showed no evidence of an isoindole (Figure 4.18). This may occur because the methyl group is too small to sterically hinder attack on the formed isoindole ring.

For butylamine in a chloroform OPA reaction mixture (Figure 4.19) a different pattern arose which might represent a partial reaction. This was thought possible because of the small multiplet at $\delta 7.1$. This can also be seen in Figure 4.18 though it is less prominent there. Chloroform had to be used as a solvent because of the limited solubility of the product in the standard methanol/buffer mixture. Isopropylamine also showed similar solubility difficulties. Little success generally was achieved using these amines in the desired conditions and little comparable to that reported by Simons was observed²⁹¹.

Figure 4.18. Proton (60 MHz) NMR Spectrum of Methylamine with OPA Reaction Mixture.

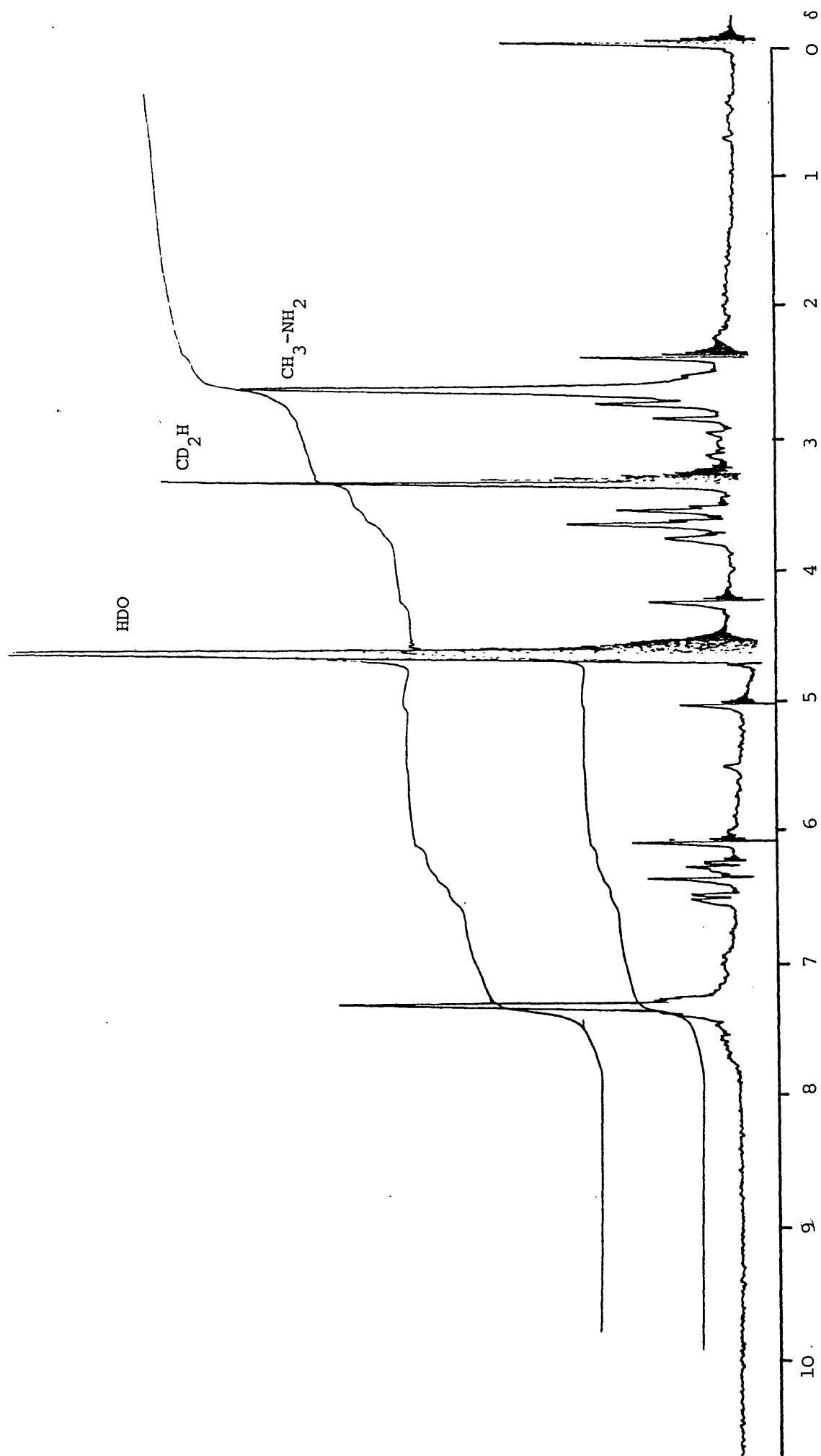
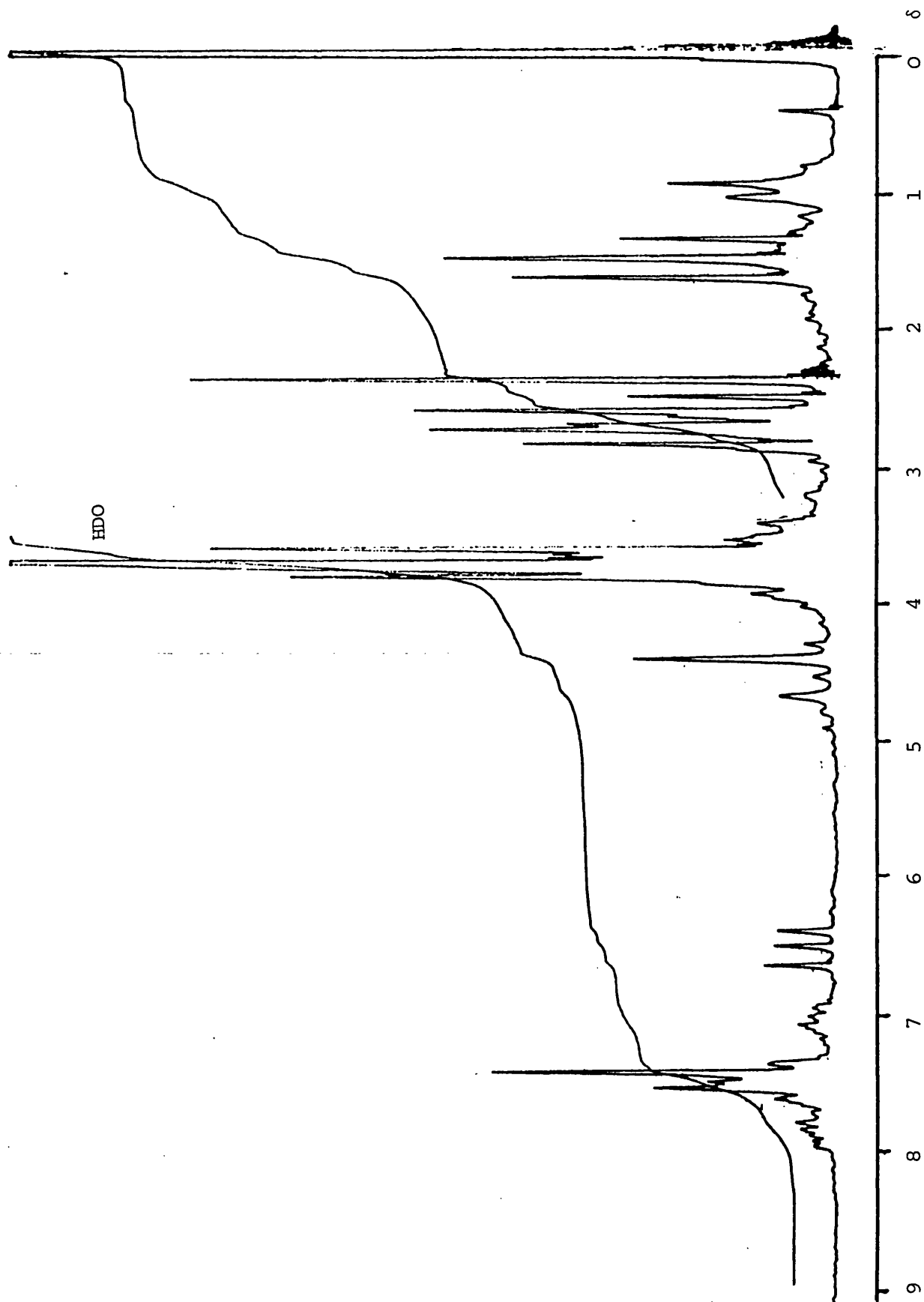


Figure 4.19. Proton (60 MHz) NMR Spectrum of Butylamine with OPA Reaction Mixture.



A potential model compound, glucosamine hydrochloride, was used in the OPA reaction owing to its structural similarity to gentamicin. Insolubility in the organic co-solvent was a major disadvantage especially at the high concentration used. Spectra produced using this amine were mostly of two types, although the circumstances for their appearance were not deduced. One type of proton spectrum was very similar to that illustrated in Figure 4.18 for methylamine and presumably represents breakdown of any isoindole formed. The other type shown in Figure 4.20 is similar to that of Figure 4.19 for butylamine. There is a multiplet at about $\delta 7.7$, downfield from the singlet for aromatic protons found in the reaction mixture. There is another multiplet at $\delta 7.1$ which may also be due to aromatic protons. The methylene group adjacent to the thiol is split which indicates that a partial reaction has occurred. It is possible that the signals at $\delta 6.7$ represent unreacted OPA, perhaps in the dihydro-isobenzofuran form. Thus it is possible that glucosamine did react to an extent with the OPA reagent, but a total reaction was never recorded, and often no reaction was adjudged to have occurred.

Ethanolamine was selected as a model for the reaction because of its suitable solubility properties. Results with this amine were more successful and reproducible. A proton NMR spectrum of a mixture of equal concentrations of OPA reagent and this amine in methanol/buffer is shown in Figure 4.21. The pattern of splitting of resonances in the aromatic region is comparable with that reported by Simons²⁹¹, but instead

Figure 4.20. Proton (60 MHz) NMR Spectrum

of Glucosamine with OPA Reaction Mixture.

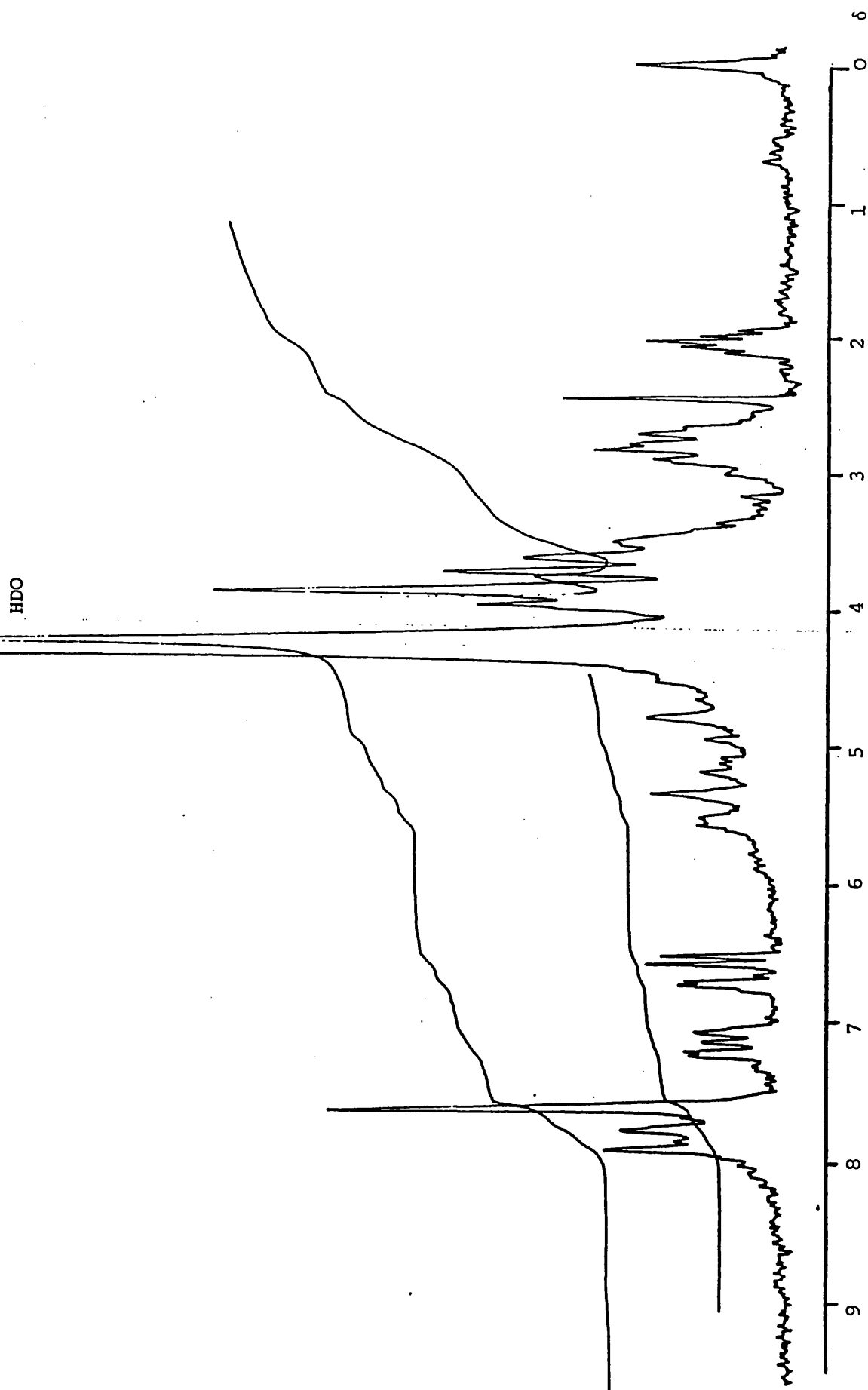
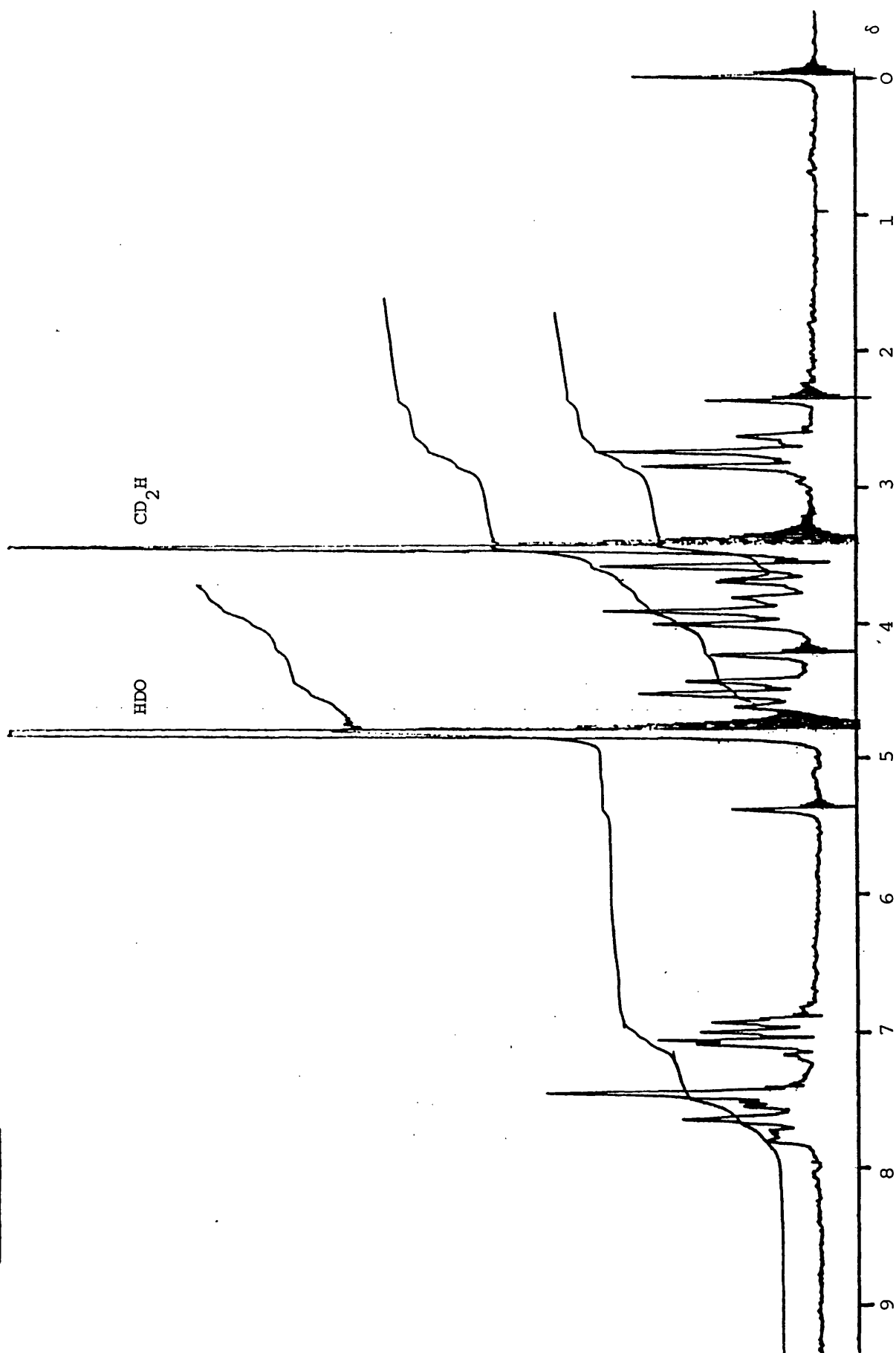


Figure 4.21. Proton (60 MHz) NMR Spectrum of Ethanolamine with OPA Reaction Mixture



of a 2:1:2 split there is a 3:2 split observed. The C-8 proton appears to have joined the lower field multiplet. The peaks in this region are also similar to that found for glucosamine in Figure 4.20, and confirms the assumption that a partial reaction had occurred. The carbon-13 NMR spectrum of the ethanolamine-OPA derivative is shown in Figure 4.22 with the shifts assigned in Table 4.5. The structure of the product is thought to be as shown in (4.VII). The high field multiplet at $\delta 6.9 - 7.2$ in the proton spectrum was assigned by Simons²⁸⁹ to the 3,4 protons (Simons; $\delta 6.8 - 7.1$), whilst the low field multiplet at $\delta 7.4 - 7.9$ (Simons; $\delta 7.4 - 7.7$) was assigned to the 2,5 protons. The proton on C-8 was found by Simons²⁸⁹ to have a shift of $\delta 7.31$, but this was thought to be at $\delta 7.4$ and part of an aromatic multiplet in Figure 4.21.

The assignment of the aromatic carbon atoms in the carbon-13 spectrum in Figure 4.22 is not absolute because of the small shift difference between them. The aromatic chemical shifts are of a similar magnitude to those reported for N-methylisoindole²⁹⁸, but the 7,8 carbons appear to be inconsistent with those of (4.VII). The thioether substituent which is electron withdrawing would be expected to deshield the C-7 carbon and this may be responsible for the peak at 121.55 ppm. However, it is unlikely to shield either the C-1 or C-6 carbon by 14.5 ppm and deshield the other (C-1 or C-6) by 7.2 ppm. More anomalous is the deshielding of the C-8 carbon by at least 6 ppm when shielding is a more usual long range effect in benzenes. For mono-substituted benzenes the

Figure 4.22: Carbon-13 NMR Spectrum of Product of Ethanolamine Reaction with OPA.

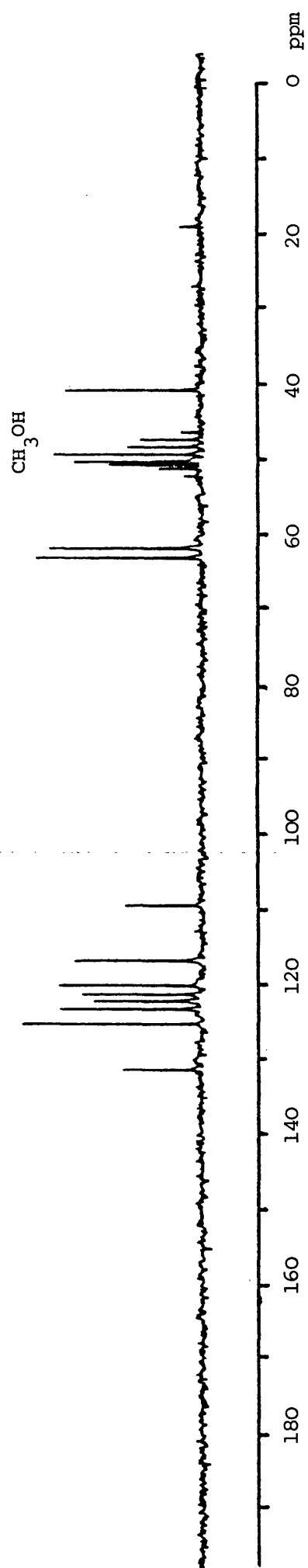
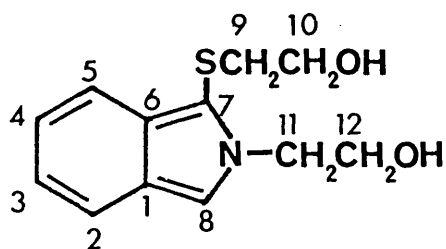
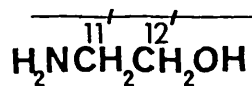
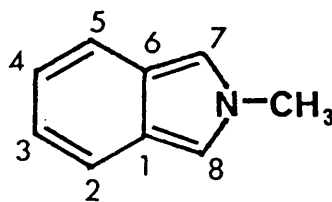


Table 4.5. Carbon-13 NMR chemical shifts of ethanolamine-
OPA reaction in comparison to N-methylisoindole.



(4.VII)



(4.VIII)

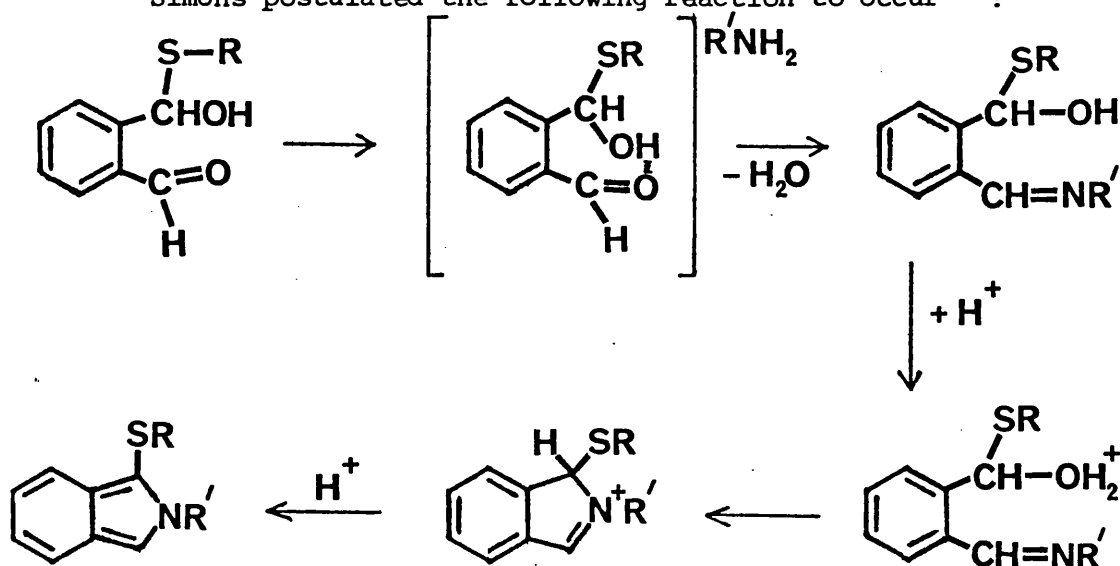
Carbon	Off Res	Chemical Shift (ppm)	Carbon	Off Res	Literature values Chemical shift (ppm)
6	s	131.47	1,6	s	124.3
4	d	125.45	3,4	d	120.2
3	d	123.50	2,5	d	119.1
5	d	122.47	7,8	d	111.3
2	d	120.37	CH ₃	q	37.1
7	s	121.55			
8	d	117.11			
1	s	109.85			
9	t	41.05			
10	t	61.96			
11	t	50.85			
12	t	63.26			
9'	t	27.00			
10'	t	64.00			
11'	t	44.20			
12'	t	63.10			

effect of ($-S-CH_3$) is only weak, apart from on the carbon of attachment²⁷³. The difference in shifts between (4.VII) and (4.VIII) appears to be too great to be accounted for by the structure (4.VII), although the lack of detailed knowledge on the effect of substitution in this ring system suggests that no definite conclusion can be reached.

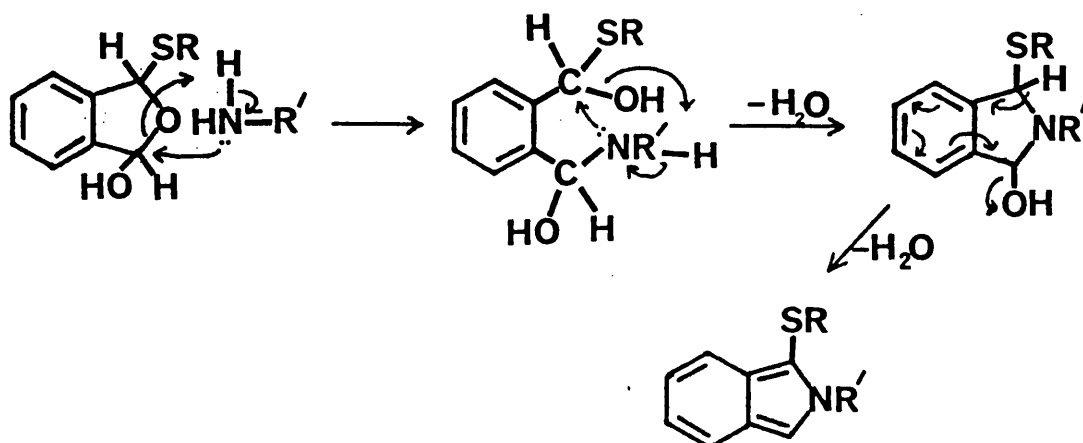
Simons²⁸⁹ did not examine the product by carbon-13 NMR, but found in the proton NMR spectra of the isoindoles that the methylene protons of C-11 were considerably shielded. A similar shielding of 7 ppm was found here for the C-11 carbon in Figure 4.22. However the C-9 carbon is substantially deshielded by about 14 ppm in respect to the free mercapto-ethanol, whilst Simons found the corresponding protons to be unaffected.

The reaction of the OPA reagent with amines was considered by Simons²⁸⁹, to proceed by the thiohemiacetal (4.IV). He thought the formation of the disubstituted, dihydro-isobenzofuran (4.V) to be a side reaction, as the concentration of it in the reagent was dependent on the thiol present. He also considered it to be unreactive towards amines especially in comparison with (4.IV). However under the conditions studied here the intermediate was found to be highly unstable and apparently susceptible to nucleophilic attack. The structure (4.IV) was not recorded by either of the NMR methods and the possibility of it reacting with the added amine seems remote.

Simons postulated the following reaction to occur²⁸⁹:



This reaction is fairly plausible except for the condition that it usually occurs at a high, alkaline pH. The primary amine nucleophilic addition reaction with carbonyl compounds, such as aldehydes and ketones, is known to produce a Schiff base if one of the substituents is aryl. However the reaction can be subdivided into two²⁹⁹; the initial addition reaction of the amine and followed by dehydration of the carbinolamine thus formed. The latter pathway requires a pH of 2 - 5, and at a pH of 10, will be very slow. Also if an imine was formed, it would probably be more liable to nucleophilic attack than the thiohemiacetal function. The reaction proposed here is:



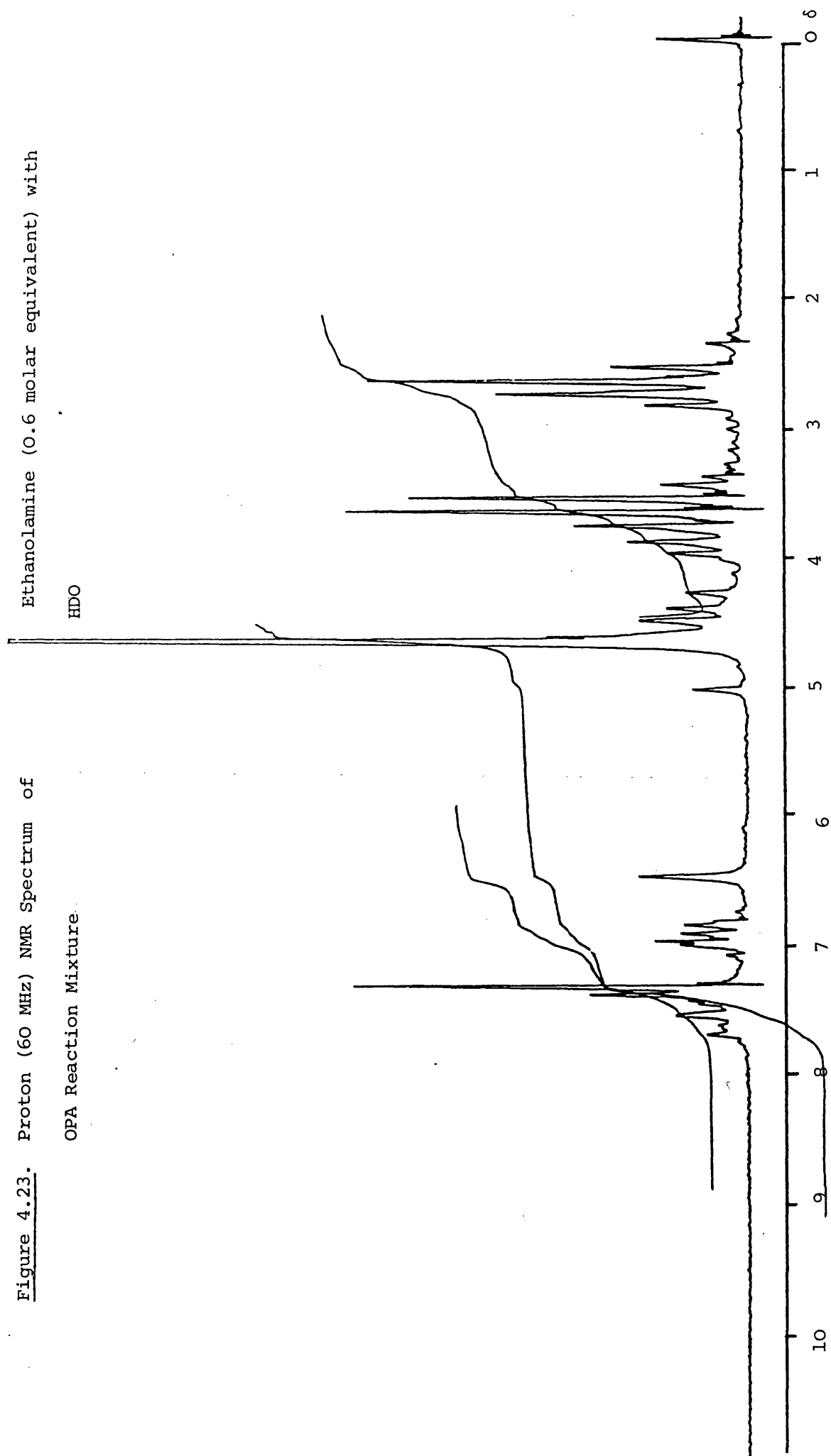
The isobenzofuran derivative is thought to be susceptible to nucleophilic attack in the conditions prevailing, and in this regard the primary amine is a more reactive agent than the thiol or other species present. The carbinolamine formed is likely to react with the electrophilic centre close to it, rather than undergo an unfavourable dehydration reaction. Once the nitrogen-containing ring is formed, dehydration of the dihydroisoindole to the more aromatic isoindole is favoured.

Without the thiol present a series of decomposition products form from the dihydroisoindole produced³⁵¹. The thiol is therefore necessary to ensure stability of the formed isoindole, presumably because it is a poor "leaving" group.

4.3.1.d Quantification of Extent of OPA Reaction BY NMR

The formation of the isoindole type product (Figure 4.21) from a reaction mixture (Figure 4.2) shows a dramatic change in the signal corresponding to the aromatic protons. A possible marker of the change in species in the reaction mixture was the two proton singlet at about $\delta 6.7$ in Figure 4.2. This signal was undisturbed by the new peaks created by the formation of the isoindole derivative, unlike the four proton singlet at $\delta 7.5$ which was overlain by the new aromatic multiplets. Quantitation of the reaction could therefore be achieved by measuring the ratio of the integral of the peak at $\delta 6.7$ to that of the newly formed higher field multiplet at $\delta 6.9 - 7.2$. In Figure 4.23 is illustrated the proton NMR

Figure 4.23. Proton (60 MHz) NMR Spectrum of
OPA Reaction Mixture.



spectrum of ethanolamine in a 0.6:1 molar equivalent relationship with OPA. This shows the singlet of the OPA reaction mixture and the newly formed multiplet of the isoindole analogue. The reaction of ethanolamine and the OPA reaction mixture over a series of concentrations from 0.2:1 to 0.8:1 molar relationships was performed to give the ratio of new aromatic multiplet to reaction mixture singlet. The four point calibration graph is illustrated in Figure 4.24. The correlation of the line is 0.9952 which indicates a significant relationship at the 0.1% level as determined by Student t-test. The slope of the line is 1.09 which is not significantly different from 1 at the 5% level.

Thus using this model, a method of estimating the extent of reaction of an amine with OPA was discovered, because there was a direct relationship between appearance of the isoindole multiplet and disappearance of the reaction mixture singlet.

The reaction of the gentamicin components did not provide spectra as sharp as those for ethanolamine. The product and reaction still exhibited the same general characteristics, but the peaks were considerably broadened. An example of a 0.75 molar equivalent reaction of gentamicin C_1 amino groups with OPA is illustrated in Figure 4.25. In this mixture the concentration of OPA was 4 times greater than the concentration of gentamicin C_1 (3 amino groups/molecule). The broadness of the aromatic peaks may be a reflection of the different amino functions reacted in the gentamicin compound. For quantitative analysis ratios of 5.33:1 and 4:1 OPA to gentamicin

Figure 4.24. Plot of Calibration of Ethanolamine Concentration
to newly formed Multiplet (PMR)

Proportion of 'NEW'
Aromatic Signal
(δ 6.9-7.2)

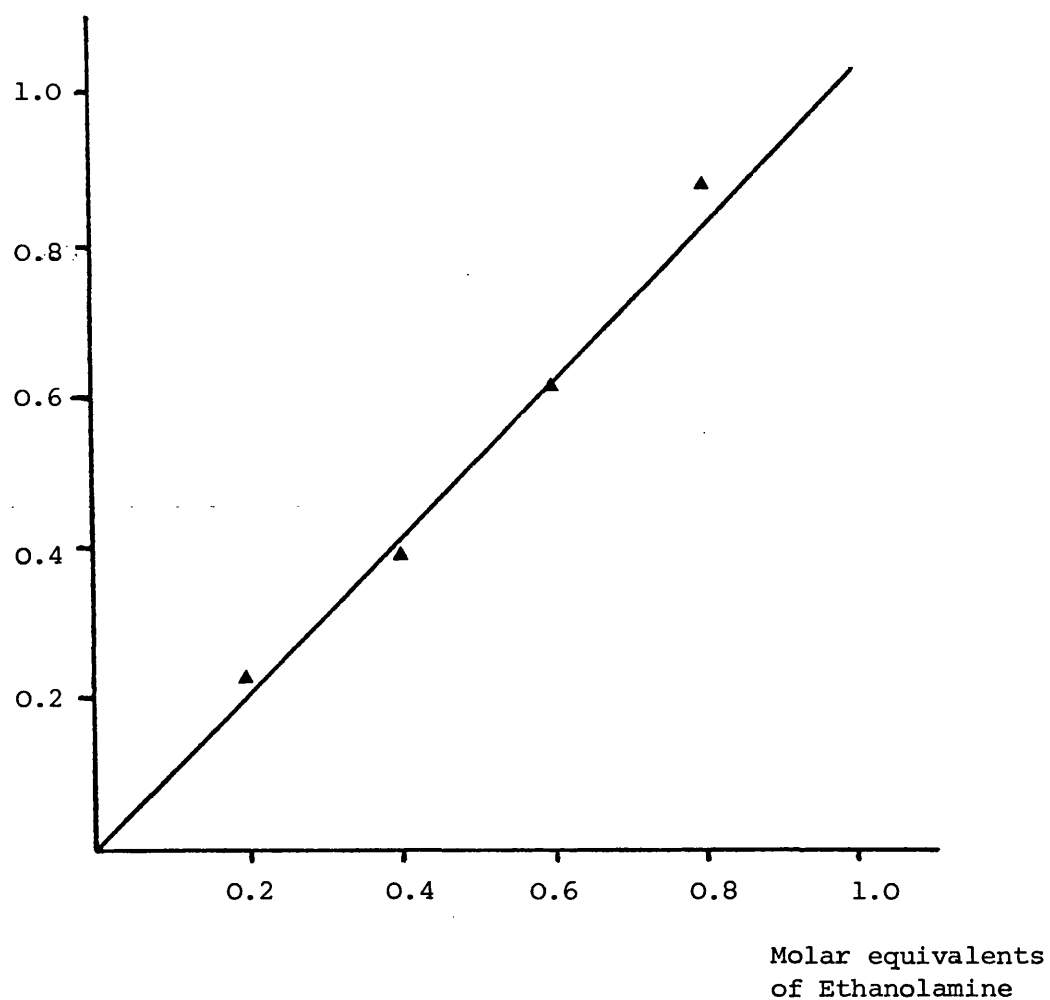
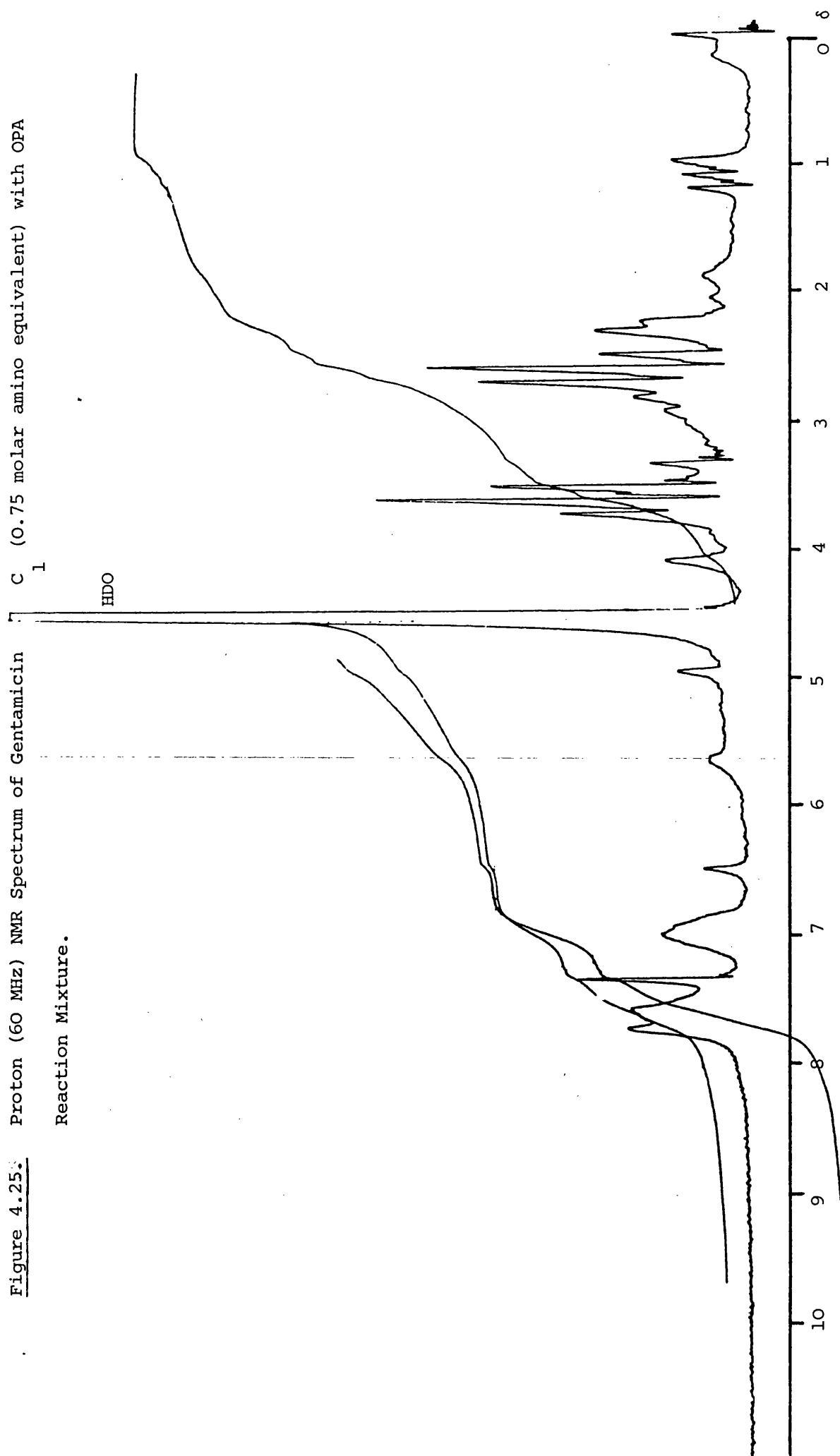


Figure 4.25. Proton (60 MHz) NMR Spectrum of Gentamicin

Reaction Mixture.



component, were used for gentamicin C_2 and C_1 respectively. This was set to achieve a concentration of 0.75 equivalents of primary amino functions in respect to 1 equivalent of OPA for both components. The quantity of reactants used was assessed by weight since it was difficult to accurately determine the concentration of gentamicin component by integrals. The fraction of reacted OPA calculated by the integral ratio, by reference to the calibration graph (Figure 4.24) is given in Table 4.6. Also included in this table is the expected amount of OPA consumed for the two components if various numbers of amino functions reacted.

Table 4.6. Stoichiometry of OPA Reaction with Gentamicin

Amino groups reacted	Expected % OPA consumed	
	Gentamicin C_1	Gentamicin C_2
1	25	19
2	50	38
3	75	56
4	-	75
Actual % OPA consumed	89	85

The actual amount of OPA found to have reacted are not significantly different from 75 at the 5% level (Student t-test), though a much closer agreement would be preferable. The table also shows that if fewer than all available amino groups had reacted then the expected amount of OPA consumed would have

been much smaller. It is fairly conclusive proof that the OPA reagent does react with all the primary amino groups in gentamicin. The apparent excess of OPA which reacted can be explained as loss in transfer of the OPA reagent to the gentamicin component. The problem of the different solubilities of the product and reactants was difficult to overcome at the high concentrations required for NMR analysis, and losses due to the enforced experimental procedure reduced the accuracy of the technique.

4.3.2 Fluorescence Measurement of o-Phthalaldehyde Reaction

To provide alternative evidence for the results obtained by proton NMR for the stoichiometry of the OPA-gentamicin reaction, the fluorescence yield obtained for each of the components was studied. It was discovered that no simple relationship between the maximum fluorescence yield and numbers of primary amino groups on the parent gentamicin component existed. An alternative measure of the reaction was thus sought. Gentamicins C_{1a} and C_2 each possess four primary amino groups and can theoretically react with four molar equivalents of OPA, whereas gentamicin C_1 has only three primary amino groups and can react with three molar equivalents of OPA. Accordingly an experiment was carried out in which the fluorescence obtained when OPA was used at a three fold molar excess was compared with that obtained when OPA was used at a four fold molar excess. With gentamicins C_{1a} and C_2 the fluorescence might be expected to fall by 25% whereas

the fluorescence of the C_1 derivative should remain more or less constant.

The percentage reduction in fluorescence observed for three different reaction times is given in Table 4.7.

Table 4.7. Reduction in Fluorescence on Changing OPA Concentration

Time (min)	% Reduction C_{1a}	% Reduction C_2	% Reduction C_1
85	32.6	42.9	35.7
110	30.4	45.2	34.0
120	32.0	48.6	34.8
Mean	31.7%	45.6%	34.8%

It is obvious that the relationship was not observed and no trend between the components could be detected. A possible explanation of this is that in the absence of an excess of OPA reagent the rate of reaction may have been reduced and may not have gone to completion. In the preparation of OPA derivatives for HPLC a substantial excess of OPA reagent is routinely used.

Due to the susceptibility of the fluorescence determination to impurities and the interpretational difficulties of results discussed above, a means of estimating the extent of reaction of the OPA reagent which was independent of fluorescence measurement was sought.

4.3.3 Combined Ninhydrin/o-Phthalaldehyde Reaction with Gentamicin

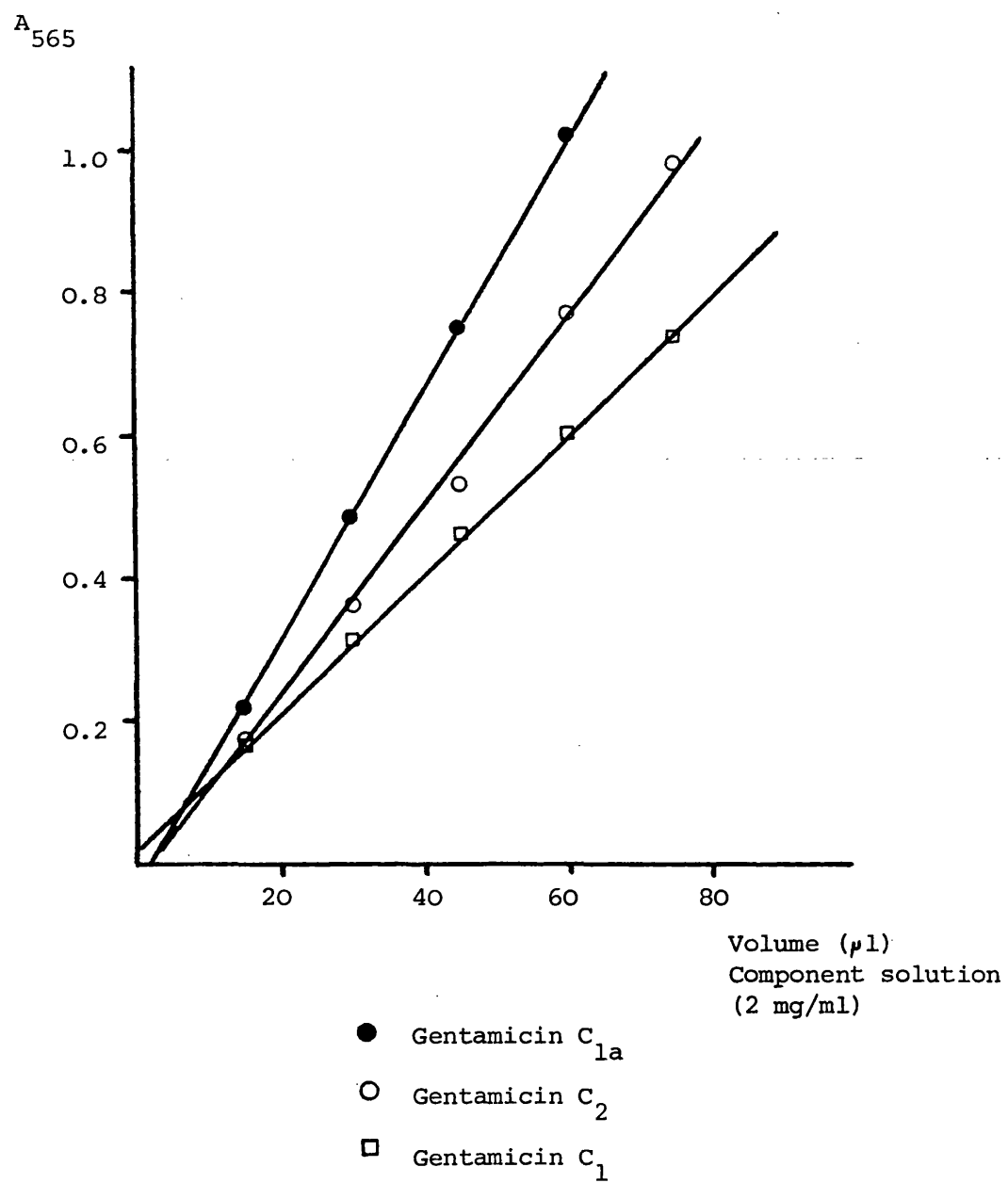
The method developed involved the construction of a calibration graph of the absorbance obtained on reacting ninhydrin with varying concentrations of the gentamicin components. Then OPA was reacted with the components in a range of concentrations and the primary amino functions left underivatised were reacted with ninhydrin. From the calibration graph already made the amount of amino groups reacting with ninhydrin could be calculated, thus giving the extent of reaction of OPA. This assumed that all the available primary amino groups would react with the ninhydrin reagent, which appears reasonable considering the reaction conditions.

A calibration graph for each component of concentration against absorbance (565 nm) was made for the reaction with ninhydrin. This plot is shown in Figure 4.26 with each component exhibiting a linear relationship of significantly high correlation. Regression analysis figures of the plots in Figure 4.26 are given in Table 4.8.

Table 4.8. Regression Analysis of Calibration Lines

	C_{1a}	C_2	C_1
r	0.9996	0.9983	0.9995
slope	0.01703	0.01310	0.00986
intercept	-0.0177	-0.0206	0.0098

Figure 4.26. Calibration of Gentamicin Reaction with Ninhydrin.

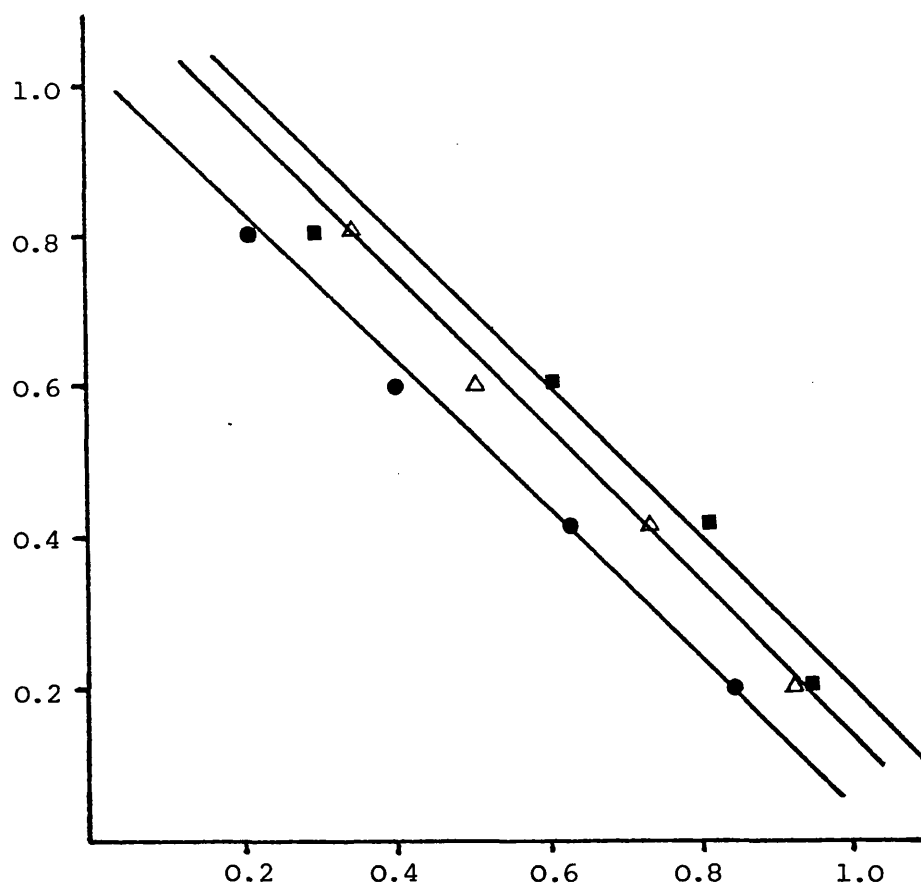


A further calibration was made to ensure reproducible behaviour of the reaction, using identical conditions, but with a freshly prepared reagent on a different day. Again a linear relationship for all three components was observed and a statistically close match of the plots resulted. There was some variation but nonetheless the ninhydrin reaction was more stable than the fluorescence reaction. However this variation required the experimental determination of the component reaction with OPA to be carried out on the same day as the calibration.

The initial concentrations of OPA chosen to react with the gentamicin was in the range of 20 - 80% of total amines present. The OPA when added, produced a fall in the observed absorbance when reaction followed with ninhydrin. The percentage of gentamicin left to react with the ninhydrin was determined by reference to the constructed calibration graph (Figure 4.26). A plot of gentamicin component remaining against the fraction of total OPA used yielded a linear plot as shown in Figure 4.27. The three components had a significant high correlation with a slope of about -1.0 for each, where OPA is expressed in terms of primary amino group equivalents. This suggests that OPA is reacting with one mole of primary amino functions per mole of OPA. The reaction was extended to 100% completion to ensure that all of the amino groups will react. A further study of the 30 - 100% concentration was thus made with the results plotted in Figure 4.28. The slopes shown are linear except when total

Figure 4.27. Plot of Effect of OPA on Reducing Available
Amino Functions

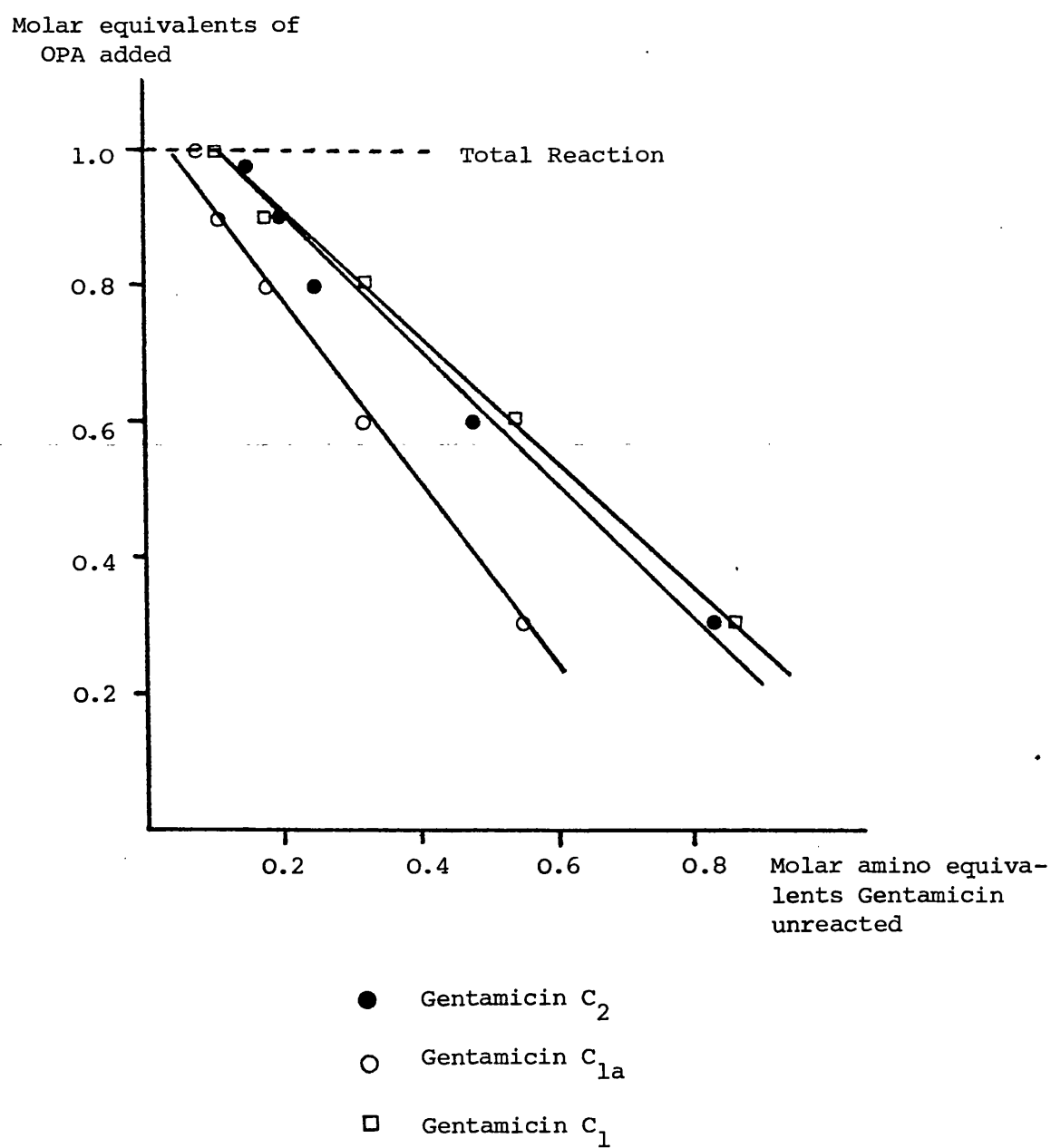
Molar equivalents
of OPA added



Molar amino equivalents
of Gentamicin unreacted

- Gentamicin C_{1a}
- △ Gentamicin C₂
- Gentamicin C₁

Figure 4.28. Plot of Effect of OPA on Reducing Available Amino Functions - extended to Total Reaction.



reaction is approached. This was due to the OPA reagent absorbing slightly at this wavelength. This absorbance was not important at low concentrations of OPA, but as 100% reaction was approached it became more dominant because of the general fall in absorbance. The plots in Figure 4.28 are not significantly different from those in Figure 4.27 at the 5% level (Student t-test). The mean of the slope of the three lines in Figure 4.28 was -0.995 indicating that the -1.0 relationship still held. Figure 4.27 illustrates that OPA is able to react with all the primary amino groups available for reaction with ninhydrin.

Assuming that ninhydrin does react with all primary amino functions on the component, then OPA can be said to react with all the primary amino groups for all of the components. This agrees with the conclusions drawn from the experiments with NMR considered previously.

CHAPTER 5

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC RETENTION CHARACTERISTICS

OF GENTAMICIN-ORTHO-PHTHALALDEHYDE DERIVATIVES

5.1 Introduction

The three components of gentamicin have shown different retention orders using very similar high performance liquid chromatographic methods^{78,223,233,234}. This is not desirable since a standard assay technique requires reproducible responses. The lack of available standards for these components only heightens this disadvantage. The o-phthalaldehyde derivative of the gentamicin C₁ component has been shown to elute either as the first^{223,233} or the last^{78,234} of the components, in HPLC systems, depending upon the paper consulted. The reaction of the fluorescent reagent with the primary amino groups, reported in Chapter 4, was studied to ensure complete and repeatable derivatisation. The lack of any potential reaction problem suggested that the anomalous retention behaviour must have arisen from the conditions used in the chromatographic stage of the respective assays. To understand the retention mechanism involved a brief summary of the possible variables will be made.

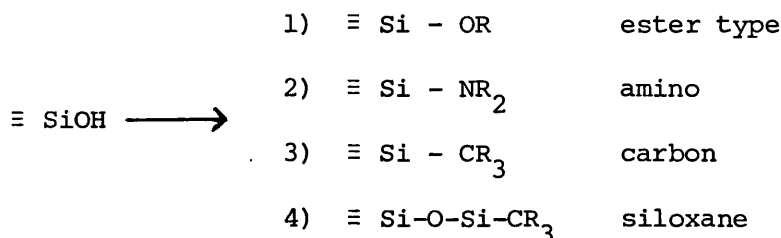
The development of HPLC has produced a technique which is sensitive, versatile, and well adapted to the analysis of typical biological and pharmaceutical compounds^{241,300}. The chromatographic processes involved in the separations are heavily dependent on the type of stationary phase used. These have been

classified as being liquid-solid (LSC), liquid-liquid (LLC), bonded phase (BPC), ion-exchange (IEC) and gel permeation chromatography (GPC)²⁴¹. The theory of the retention mechanisms in LSC, LLC and GPC are fairly well understood but doubt exists as to the exact mechanism in ion-exchange or bonded phase chromatography²⁴¹. In the case of IEC the uncertainty arises mainly because other factors apart from ion-exchange are thought to be involved. In BPC the nature of the retaining phase is the principal source of the ambiguity³⁰¹⁻³⁰⁵.

Bonded phases were used in the gentamicin assays, and indeed BPC is the most widely used method in HPLC and has the widest range of commercial packings³⁰⁶. It can be termed either normal or reversed phase chromatography, depending upon the functional groups which are attached to the silica microparticles²⁴¹. Normal phase packings have groups such as diol, dimethylamino, nitro, cyano and amino bonded to the silica surface³⁰⁶. These groups confer differing properties to the stationary phase which is used with mobile phases similar to those used for silica. The groups used for reverse phase packings are more hydrophobic, being straight chain paraffins, and include methyl, octyl and octadecyl functions³⁰¹. These groups produce a hydrophobic surface, unlike the highly polar silica surface, and the solvent strength of eluting solvents is reversed³⁰⁷.

The bonded groups are formed after reaction at surface silanol groups (SiOH) through a number of bridging functions

shown^{302,303}:



The siloxane type is the most frequently used in reversed phase BPC and are synthesised using an organohalogenosilane³⁰³. This can be either mono, di, or tri-alkyl substituted and usually a chlorosilane is used. The monochlorosilanes such as dimethyloctadecylchlorosilane produce a monomeric bonded phase on the silica and are the most frequently used³⁰². With di and trichlorosilanes some of the chloro groups will be unable to react with the surface silanols and are free to react with other reagent molecules^{305,306}. A polymerised layer is thus produced which can shield the silica surface from dissolution in severe conditions of use, such as high pH. However these phases exhibit slow mass transfer of solutes which decreases efficiency³⁰¹. Batch variability is another drawback to this type of phase³⁰⁶.

The actual conformation of the bonded groups will ultimately depend on the position and number of silanol groups on the silica surface³⁰⁵. These factors are dependent on the manufacturing process and conditions used to produce the silica microparticles³⁰². The silanols are randomly distributed on the surface and can be described as being paired or isolated^{303,310}. The paired hydroxy groups are hydrogen bonded to each other and are less

than 0.31 nm apart. The isolated hydroxyls are separated by a mean distance of 0.65 nm and are more exposed for reaction³⁰³. The ratio of the two types of silanols will dictate the extent of the reaction with chlorosilane. An estimate of the surface coverage of free silanols is of about $8 \mu\text{mol m}^{-2}$ ^{303,309}. Maximum reaction with these groups with a mono-chlorosilane has been $4.5 \mu\text{mol m}^{-2}$ for trimethylchlorosilane and $2.36 \mu\text{mol m}^{-2}$ for n-hexyldecadimethylchlorosilane³⁰³. Thus although a 100% coverage of the surface with the trimethylsilane will have occurred, only 60% of the surface silanols have reacted. The residual silanols are believed to be inaccessible to solutes, either from steric hindrance by the organic layer or due to the location of the silanols in micropores³⁰³. The size of the organic bristle is an important consideration, especially in relation to pore size, when contemplating the effect of the bonded phase^{302,304}. It is the pores of the silica particles which are responsible for the high specific surface area associated with the packing. Silica, with a particle size of 10 μm and a specific surface area of $300 \text{ m}^2 \text{ g}^{-1}$, has an external surface of only $0.3 \text{ m}^2 \text{ g}^{-1}$ with the remainder residing in the pores³⁰³. The diameters of these pores are mainly in the 5 - 12 nm range. The length of an octyldecyl (ODS) group is about 1 nm³⁰³, and thus complete reaction should decrease the diameter by about 2 nm. This will thus decrease the surface area and pore volume, depending on the diameter of the pore³¹¹, and such a reduction can be up to 50%³⁰². However, this has not always been confirmed by gas adsorption experiments³⁰³.

Since the large organic reagents do not always react to completion, a small chlorosilane is often used to cover any remaining silanols. This procedure is called "endcapping" and helps produce a more homogenous phase³⁰⁸. Tests for residual silanols are usually carried out either by determining methyl red adsorption by the support or by measuring the retention of a polar solute on a column of the support using a non-polar solvent as eluent^{304,306}. By the first method a silanised material shows no red colour and by the second a capacity factor of less than 0.5 should be observed. Any residual silanols are believed to decrease the efficiency and stability of the column^{301,308}.

General statements on the retention behaviour of compounds in reversed phase BPC can be made even though the exact mechanism is not understood. If the length of the hydrocarbon chain of the bonded phase is increased the retention will also increase^{241,301,304}. The solvent strength of the eluent is the inverse of that found in LSC on silica^{241,312}. The mobile phase consists usually of a binary mixture of water and an organic modifier. A plot of the percentage modifier present against the logarithm of the capacity factor (k') (3.2.2.a) is usually linear^{313,314,307}.

As with other forms of HPLC an increase in temperature of the column will reduce the k' value^{311,315-17}. This is because the thermodynamic distribution constant (k^0) is related to temperature by:³¹⁸

$$\ln K^O = - \frac{\Delta H^O}{RT} + \frac{\Delta S^O}{R}$$

At low concentrations of solute the thermodynamic distribution constant (K^O) is equal to the concentration distribution coefficient (K)³¹⁹

$$\text{and} \quad \kappa' = K \left(\frac{V_x}{V_y} \right)$$

$$\ln \kappa' = - \frac{\Delta H^O}{RT} + \frac{\Delta S^O}{R} + \ln \phi$$

where ϕ is the phase ratio $\left(\frac{V_x}{V_y} \right)$ of mobile and stationary phases.
 (V_y) (V_x)

A linear relationship is therefore expected between $\ln \kappa'$ and the inverse of temperature with the slope equal to the change in enthalpy on binding^{315,317}.

Compounds which belong to a homologous series can also show a linear relationship between $\ln \kappa'$ and the number of functional groups present. From this independent estimates of group contribution to retention can be made (3.3.1)^{241,317,318}.

The mode of retention in BPC has been described as being either by adsorption or by partition³⁰²; the two major mechanisms in chromatography. The fixed ODS chains in BPC restrict conformational freedom, unlike the corresponding apolar liquid in conventional partition systems³¹⁵, whilst the adsorptive properties of the ODS groups are only weak when compared to such adsorbents as alumina or silica³²⁰. The driving force for retention is therefore unlikely to

be entirely due to one of these processes solely and some other factor must be involved. Locke³²⁰ determined that elution order in BPC with alkyl side chains was in inverse relation to the solute solubility in the mobile phase and that selectivity was due entirely to the eluent.

A more comprehensive expression of retention has been the development of the Solvophobic theory by Horvath and co-workers^{307,312,321}. The solute is assumed to bind to the non-polar phase with a free-energy change associated with the binding. The magnitude of this change is the difference in energy between bringing the bound complex into solution and placing the individual components into the solvent. The main determinants of the free energy of binding are the surface tension of the solvent, the molecular contact area taken up on binding of the solute, and electrostatic and van der Waals interactions between solvent and components of binding. For unionised solutes the largest effect is due to surface tension; an increase of which leads to an increase in retention time³²¹. For ionised solutes there is a more complex relationship since the electrostatic terms are more important.

However, anomalous retention of ionic compounds has been observed which cannot be explained by the solvophobic theory^{311,313,322-30}. This is readily apparent in a plot of $\ln k'$ against percentage organic modifier in which ionic compounds often show a curve whereas the theory would predict a linear relationship^{313,322,328,329}. This effect can be

reduced by suppression of ionisation by adjustment of the pH of the eluent³⁰⁸. However for a base with a pKa of 9 the pH of the mobile phase would have to be at least 9.5, and under such conditions the lifetime of the column would be short. Another method is to use alkylamines which can restore regular retention behaviour^{313,326-32}. The apparent discrepancy with ionic compounds in reversed phase BPC has been attributed to a dual site mechanism of retention^{311,330,327}. Various models have been proposed to account for the two sites^{327,330,331}, which are generally believed to be the ODS chains and the residual silanols present.

Another method for minimising the possible influence of the silanols on ionic compounds is to interact the solute ions with ions of opposite charge dissolved in the solvent^{317,333-43}. These counter ions usually possess hydrocarbon chains and often are surfactants³³⁹. Several mechanisms have been proposed to account for this effect³³⁴.

- i) Ion-pairing of the ions in solution followed by adsorption on the ODS packing^{317,331,333,336}
- ii) Dynamic ion-exchange of the solute with the adsorbed counter ion on the ODS phase^{326,340,342,343}
- iii) Ion-interaction mechanism, with an electrostatic double layer formed on the surface of the particles attracting the solute by electrostatic force^{334,337,338}.

It is possible that any or all of these may occur depending

upon the hydrophobicity of the counter ion³³³. Ion-pairing may well overlap other forms of BPC since ion-interaction will occur to some extent with any ions which may be present in the mobile phase such as buffer ions.

The relationship between retention of a solute in BPC and its retention on silica is also important in understanding the chromatographic behaviour of the differing samples. Under certain conditions the behaviour of amines on unmodified silica is similar to that observed on ODS bonded silica^{330,344,345}. Some authors regard this as evidence that silica itself can behave as a reverse phase material with the hydrophobic siloxane groups exerting an influence^{330,344}. On the other hand others suggest that the mechanism on ODS bonded packings involves ion-interaction with the silanol groups³⁴⁵.

In applying these factors to the separation of gentamicin components, the nature of the stationary phase is of prime importance especially with respect to the presence of silanol groups.

5.2 Materials and Methods

5.2.1 Materials:

Spherisorb ODS, 5 μm packing material was obtained from Phase Separations Ltd., Zorbax ODS, 7 μm , silica was from DuPont (UK) Ltd. and Hypersil ODS, 5 μm was obtained from Shandon Southern Ltd. Gentamicin components had been

separated by column chromatography, and netilmicin sulphate was a gift from Kirby-Warrick Pharmaceuticals Ltd. Potassium iodide, potassium nitrate, potassium thiocyanate, sodium nitrate, sodium iodide, barium chloride and lithium chloride were all supplied by BDH Chemicals Ltd. Acetic acid (glacial) and ammonia (0.88 gr) were supplied by Fisons Scientific Ltd.

HPLC instrumentation was the same as that used in (3.2.1). Columns used were all of 150 x 5 mm dimensions.

5.2.2 Methods:

5.2.2.a. Effect of Temperature on Retention:

Reaction mixtures injected contained gentamicin (0.4 mg/ml) standard solution (50 μ l), netilmicin (0.5 mg/ml) (20 μ l) with OPA reagent (0.5 ml) and methanol (1 ml). Mobile phase was 80:20, methanol:water with 2 g/L EDTA, tri-potassium salt. The temperature of the water bath, in which the column was immersed, was varied in steps of 5° from 20° to 45°C. At each temperature at least three determinations of retention time of the gentamicin components and netilmicin were made. Individual components were also injected separately to identify peaks. The position of the non-retained solute was taken as the initial leading edge of the solvent front for all injections.

5.2.2.b Effect of pH on Retention:

Ammonia-Acetic Acid Buffer

Ammonia and acetic acid solutions of concentration of about 0.017 M were prepared by diluting 1 ml ammonia (0.885g) and acetic acid (glacial) (1 ml) to 1000 ml with distilled water. The strength of the ammonia solution was determined by neutralising an aliquot with excess 0.01 M hydrochloric acid and back titrating with a 0.01 M sodium hydroxide solution using methyl red as indicator. The strength of the acetic acid solution was determined by titration of a solution with 0.01 M sodium hydroxide using phenolphthalein as indicator. The strengths thus found were 0.018 and 0.014 M for acetic acid and ammonia respectively. Using these solutions a series of buffers were prepared ranging from pH 3.5 to 10.5 with potassium iodide added to give a constant ionic strength of 0.075 M for all solutions. Methanol (80 ml) was added to 20 ml of each of these buffer solutions and each was used as the mobile phase. The column was allowed to equilibrate with each mobile phase for fifteen minutes before injection. At least three injections of all components were made at each pH.

5.2.2.c. Addition of Salts to the Mobile Phase

Mobile phase (300 ml) of 80:20 methanol:water composition was prepared with an initial concentration of salt present. The column was allowed to equilibrate for at least fifteen minutes before injection of the gentamicin components. After at least four injections the mobile phase was changed

by dissolving in the same solution a measured quantity of salt. Volume of mobile phase was determined by measuring cylinder. Therefore the same mobile phase was used throughout for one individual salt with up to eight different concentrations of the salt used. At the end of each day, columns were washed with methanol to prevent halide attack of stainless steel fittings. No buffer was used but the pH remained about 6.5. Salts used in these determinations were EDTA, tri-potassium salt; potassium iodide; potassium nitrate; potassium thiocyanate; sodium iodide; sodium nitrate; lithium chloride and barium chloride. The range of concentrations used depended on the effect on component retention. Three different reverse phase packing materials were compared in this study; Zorbax ODS, Hypersil ODS and Spherisorb ODS. The Spherisorb and Hypersil columns were thermostatted at 30°C whilst the Zorbax column was kept at 44°C.

5.3 Results and Discussion

5.3.1 Effect of Temperature on Retention

The capacity factor (κ') was measured for each of the gentamicin components (C_1 , C_{1a} and C_2) and netilmicin at various column temperatures on the Spherisorb column. A plot of κ' against column temperature is shown in Figure 5.1. The relative retention of the components does not seem to be affected by temperature variation and the shape of the curves is as expected with the distribution coefficient decreasing with increasing temperature. As the logarithm of

Figure 5.1. Effect of Temperature on Retention of the
Gentamicin Components

Conditions: Spherisorb Column; mobile phase, Methanol/
Water (80:20); I = 0.05 w.r.t. aqueous,
with Potassium Iodide

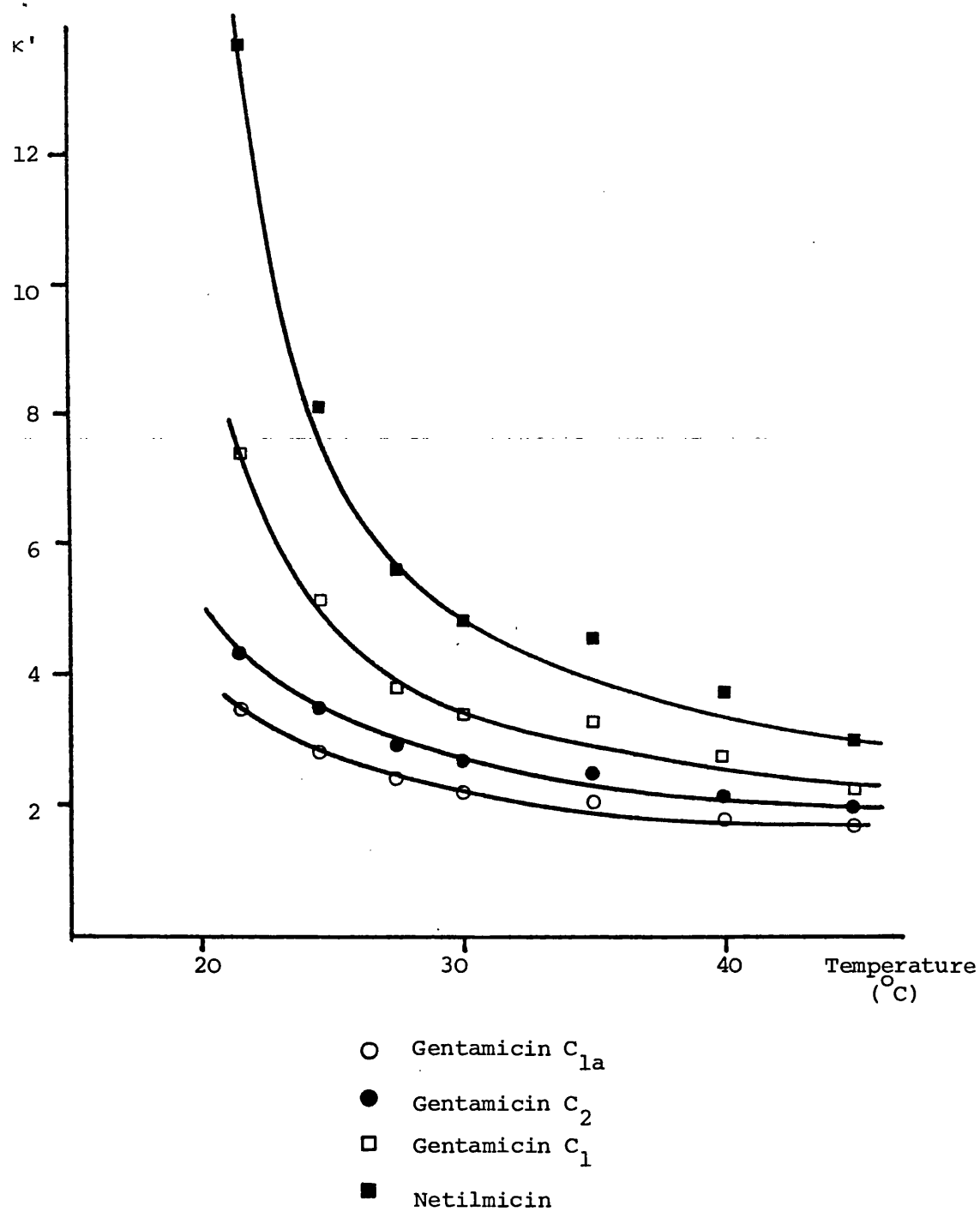
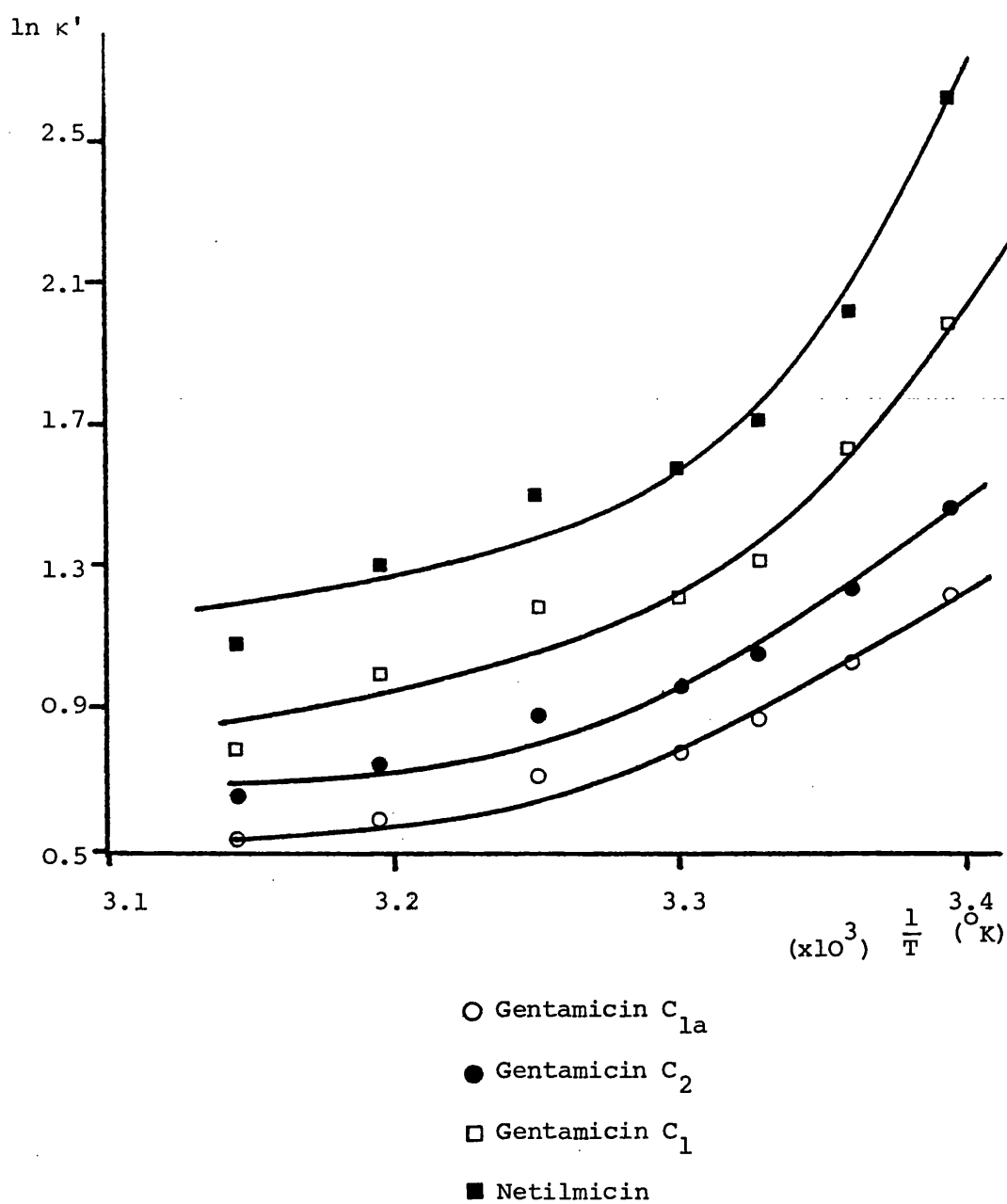


Figure 5.2. Plot of the logarithm of k' against the reciprocal of Temperature

Conditions: As in Figure 5.1.



κ' is linearly related to the reciprocal of temperature ($^{\circ}\text{K}$) a graph can be made of this relationship. The plot of Figure 5.1 adjusted to these terms is shown in Figure 5.2 with regression analysis given in Table 5.1.

$$\ln \kappa' = - \frac{\Delta H^{\circ}}{RT} + \frac{\Delta S^{\circ}}{R} + \ln \phi$$

Table 5.1. Regression analysis of $\ln \kappa'$ against $1/\text{Temp}$

	Gent C_{1a}	Gent C_2	Gent C_1	Netilmicin
(r)	0.955	0.962	0.935	0.928
slope	2584	2994	4121	5280
intercept	-7.66	-8.82	-12.21	-15.63

From the correlation coefficient (r) and Figure 5.2 it is apparent that the fit is not completely linear with a tendency to a curve, especially seen with netilmicin. The enthalpy of binding must be varying with temperature, which may be because of a two site adsorption with different adsorptive properties as suggested by Nathum³¹³. A rough estimate of the ΔH° from the graph is given for each component.

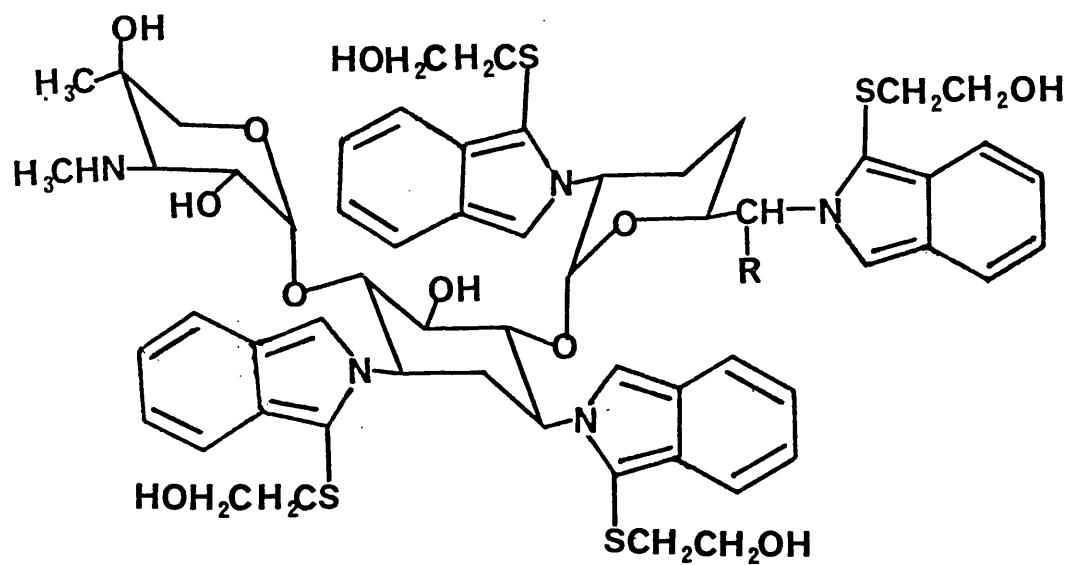
Gentamicin C_{1a}	22 KJ mol ⁻¹
Gentamicin C_2	25 KJ mol ⁻¹
Gentamicin C_1	34 KJ mol ⁻¹
Netilmicin	44 KJ mol ⁻¹

These figures suggest that gentamicin C_{1a} and C_2 are bound in a similar fashion whilst the binding of gentamicin C_1 and netilmicin may involve an extra factor since the energies are higher.

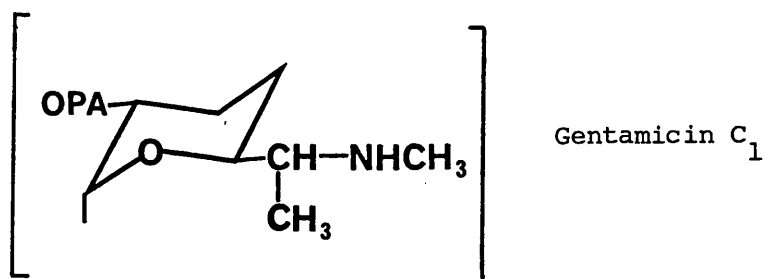
5.3.2 Effect of pH on Retention:

If the gentamicin components exhibited unusual chromatographic behaviour due to differing ionisation profiles it would be expected that pH would be an important indicator of such variation. From studies on the ortho-phthalaldehyde reaction the primary amino groups were believed to be totally reacted to form isoindole derivatives and thus not be liable to protonation (Figure 5.3.) However, the secondary amino groups should be unaffected by the reaction and therefore potential sources of ionisation. The pKas of the primary amino groups of gentamicin are about 8.2³⁵⁴ but those of the secondary amino groups have not been well established. One report³⁴⁶ gives the value as 6.2 which is markedly different from the primary amino figure. This may correspond to the secondary amino function in the garosamine ring which is situated between two carbon atoms bearing hydroxy groups. As well as inductive effects, hydrogen bonding could occur, reducing the base strength of this group. In contrast the additional secondary amino functions in netilmicin and gentamicin C_1 do not possess such neighbouring groups. Thus a pKa of about 9.0 is envisaged since the basicity of a secondary amino group is usually slightly greater than that of a corresponding primary function.

Figure 5.3. Proposed Structure of Gentamicin-OPA Derivative



R - H Gentamicin C_{1a}
 R - CH₃ Gentamicin C₂



In this experiment the buffer solution was devised to have a wide buffering range with low ionic strength and simple ions in solution. The buffering capacity was low in the middle of the range (pH 5.5 -8.5) but adequate for the purpose of investigation. The ionic strength of the buffer was calculated from the equation:

$$I = \frac{1}{2} \sum (C_i Z_i^2)$$

C_i is the concentration of each ion

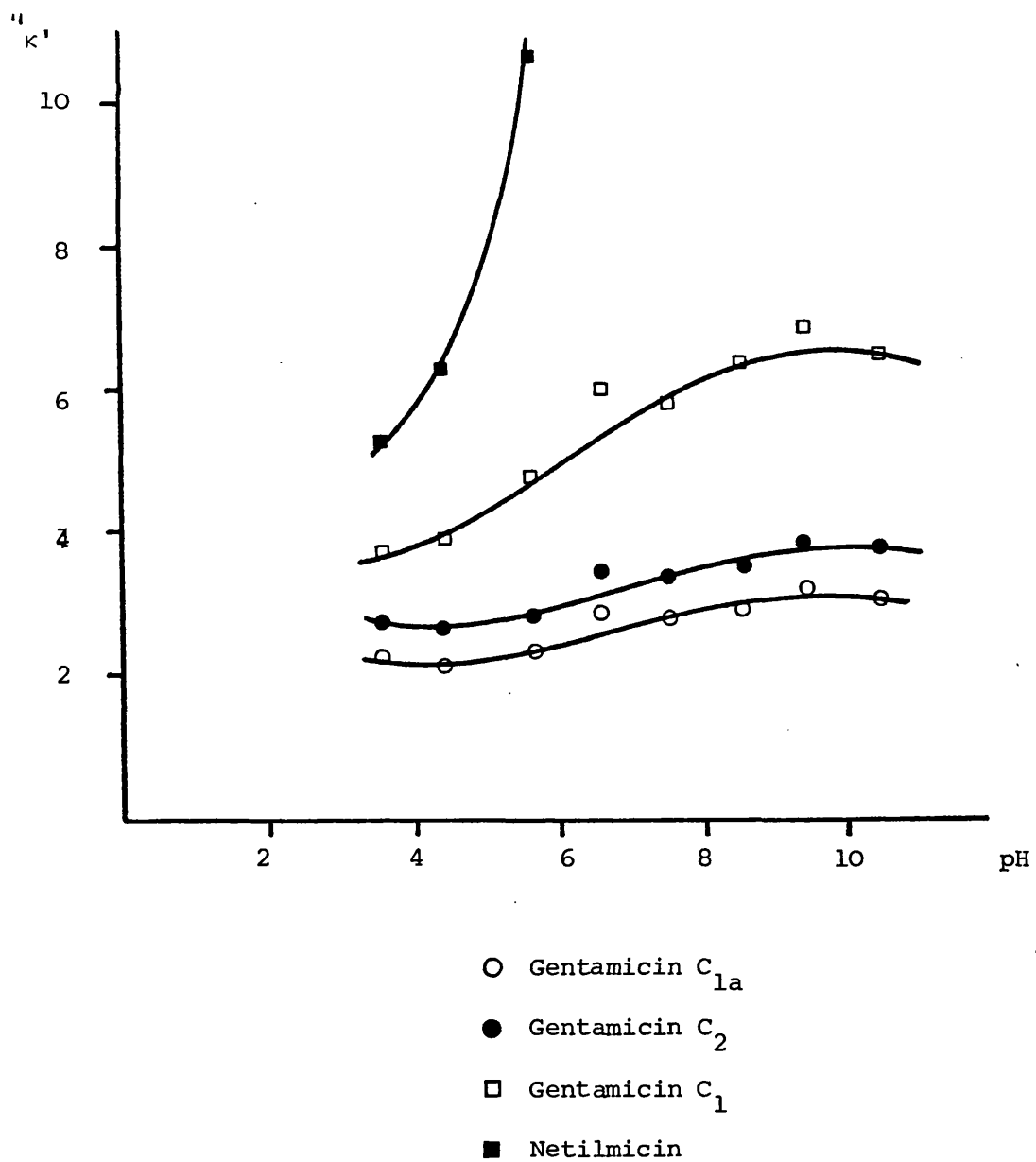
Z_i is the valency of each ion

The maximum ionic strength of the buffer, at a pH of 8.5, was 0.001 and potassium iodide was added to keep the ionic strength constant at 0.015. A pH against κ' graph for all compounds was constructed using a series of 80/20 methanol/water mobile phases on a Spherisorb column. The graph is shown in Figure 5.4 and the pH plotted is that measured in aqueous solution before addition of methanol. This was to relate all factors to a standard state (aqueous) since the ionisation behaviour of the amino functions of interest, was unknown, when in an environment such as the mobile phase.

From Figure 5.4 it is apparent that the components behave differently with respect to change in pH. Gentamicins C_{1a} and C_2 show a slight sigmoid curve with increase in pH. The curve for gentamicin C_1 is more pronounced, whilst that for netilmicin shows a dramatic increase in retention with increase in pH. The retention characteristics expected for an ionised/unionised

Figure 5.4. The variation of Retention with change of pH of the Mobile Phase.

Conditions: Spherisorb Column; Mobile Phase - Methanol/
Water; (80:20); $I = 0.075$ w.r.t. aqueous,
with Potassium Iodide. pH Measured in
aqueous fraction.




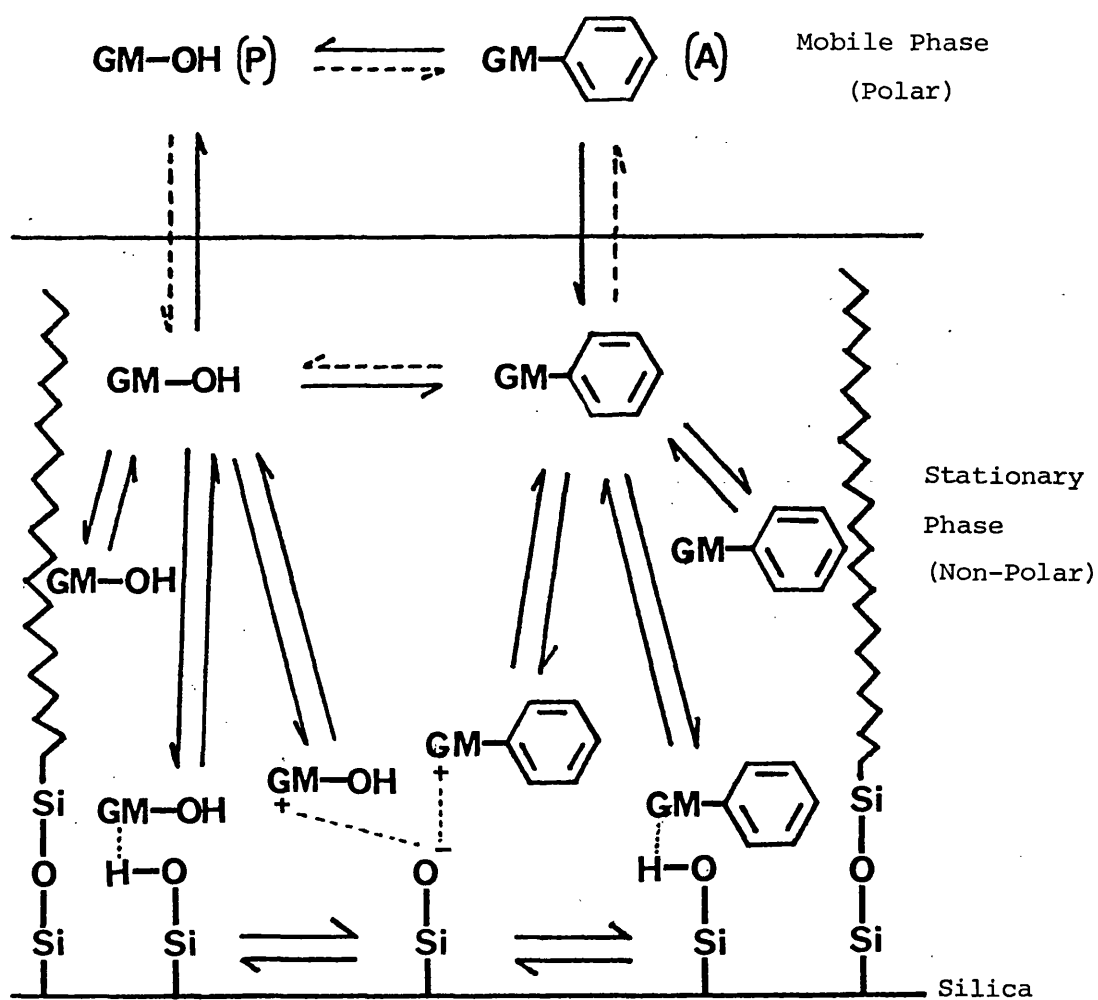
equilibrium in reverse phase BPC is a sigmoid curve³³⁴ of the type seen for gentamicin C₁. The ionised form is retained the least because of the greater solvation experienced. If ionic or hydrogen-bonding interactions were occurring with the residual silanol groups, then the pH curve would be reversed, with the ionic form being retained the most. To explain the curves found in Figure 5.4 account has to be made of the conformational characteristics of the aminoglycosides. The presence of the two glycosidic bonds in the molecule, around which the subunits can rotate, create a very flexible compound. The conformation of the gentamicin-OPA derivative can be changed to match that of the immediate environment. In Figure 5.3 is illustrated the position of the planar isoindole functions. The C-ring is free of such groups and it is relatively more polar than the other rings. By rotation the compound could possibly either present all isoindole groups to the outside, with polar functions hydrogen-bonded to the interior, or else the polar groups can be facing the exterior with the non-polar functions partly hidden. However the secondary amino group in the A ring in gentamicin C₁ may disrupt this micelle type separation of groups if it cannot be grouped with the other polar functions. The two types of conformer would be expected to be in equilibrium in both the mobile and stationary phases as shown in Figure 5.5, where GM-OH (P) is the polar conformer and GM- (A) is the apolar form. There are two other types of equilibria occurring apart from that between conformers. There is the equilibrium of distribution between the mobile and stationary

Figure 5.5. Equilibrium of Gentamicin-OPA Conformers
in Reversed-Phase Systems



phase, and also the equilibrium between binding sites on the stationary phase. The position of the equilibrium of the latter may be influenced by pH due to the ionisation of the silanols. These are known to be capable of ion-exchange³⁴⁷ and are thought to have a pKa of about 7.1³⁰³. Weak ion-exchange can occur in neutral solutions and in the range of pH 2 - 6. However maximum capacity is only attainable at high pH values (9 - 11) where the sorption capacity of multivalent cations is considerably enhanced³⁰³. Thus as the ion-exchange capability of the silanols increases with increasing pH the ionisation of the secondary amine groups decreases. The two effects might counteract and therefore not upset the equilibrium of binding to a great extent.

In such circumstances the dominant effect on change of pH will be determined by the distribution equilibria. The gentamicins C_{1a} and C_2 if they are able to successfully mask the ionic charge in conformer A will not alter the equilibrium between the mobile and stationary phases, from that observed with the unionised form. On the other hand, in the case of gentamicin C_1 and netilmicin, the exposed ionisable group in conformer A would significantly alter the equilibrium. The gentamicin C_1 and netilmicin derivatives would be expected to show a large decrease in retention on ionisation compared with little effect for the gentamicin C_{1a} and C_2 derivatives. This is borne out by Figure 5.4.

It is noticeable that on this column, with a constant

salt concentration, the relative order of retention of the components did not alter when the pH was varied. It suggests that the gentamicin C_1 and netilmicin derivatives were more strongly bound to the stationary phase than either gentamicin C_{1a} or C_2 in both the ionised and unionised form. The most likely explanation is the greater basicity of the additional amino groups the components possess.

5.3.3 Effect of Salt on Retention

The effect of salts on the retention of the gentamicin derivatives was investigated because an impure water source was observed to affect the order of retention when used in the mobile phase.

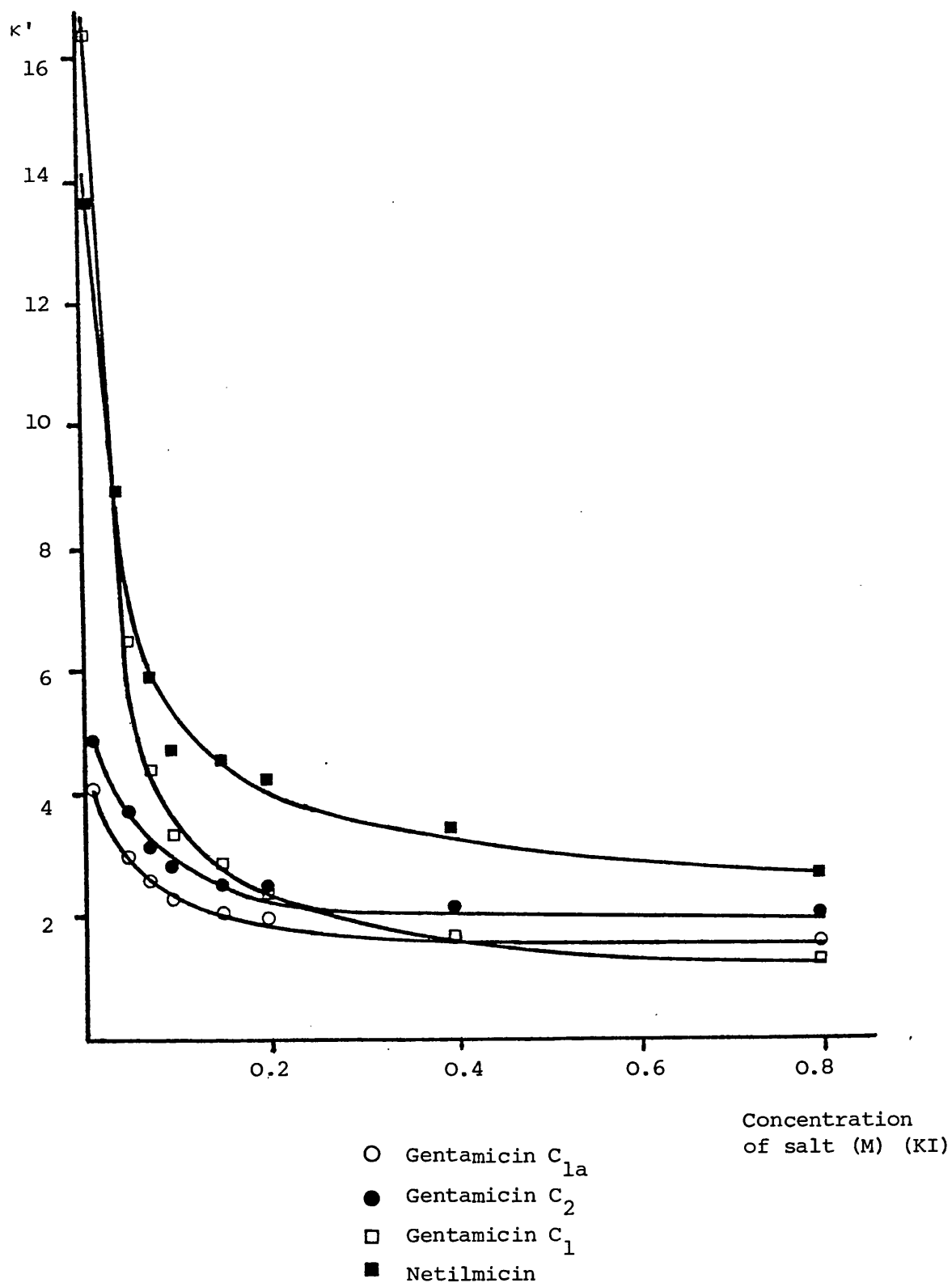
The solvophobic theory³¹² predicts a linear increase in the retention of a neutral solute as the ionic strength increases, because of the corresponding progression in surface tension. The surface tension of the eluting solvent is proportional to the concentration of the electrolyte, and a plot of $\ln \kappa'$ against I is linear. With ionised solutes, the electrostatic effects associated with the salt influences retention as well as the surface tension. The reduced electrostatic repulsion between solutes at low ionic strength dominates over surface tension and an initial fall in κ' occurs. This is at a minimum at $I = 0.3$, with about a 25% decrease in retention³²¹, and after this the retention increases due to the surface tension effects. The general increase in retention with

surface tension can be explained by an increase in the free energy of cavity formation in the mobile phase.

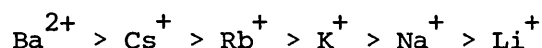
The concentration of potassium iodide in the mobile phase was varied from 0.05 to 0.80 M (in respect to the aqueous fraction) using the 80:20, methanol-water, mobile phase and a Spherisorb column. A plot of the κ' value for each of the components against concentration of salt is given in Figure 5.6. In the absence of salt no peaks were observed for any of the compounds indicating that they were irreversibly bound in those conditions. The figure shows the retention behaviour of the gentamicin C_{1a} and C_2 components to be similar yet the netilmicin and gentamicin C_1 components show different curves. The gentamicin C_1 derivative elutes after the two other components at low ionic strength, crosses the C_2 derivative at about 0.2 M and the C_{1a} derivative at about 0.4 M. The netilmicin derivative shows a nearly parallel profile to that of gentamicin C_1 . The reduction in κ' value of the gentamicin C_1 was by about a factor of 16 over this concentration range, which cannot be accounted for by the solvophobic theory. However, the resemblance of Figure 5.6 to retention behaviour on ion-exchange columns³⁴⁸ raises the possibility that ion-exchange is occurring at the silanol groups. Accordingly, a more thorough investigation of the nature of the electrolyte species on retention was carried out. For a cation exchanger the nature of the cation used in the mobile phase is important, with selectivity dependent on several factors. The ion-exchanger has a high affinity for ions of higher valence, with greater polarizability and with a smaller

Figure 5.6. Effect of Potassium Iodide on Retention.

Conditions: Spherisorb Column; Mobile Phase as previous, Salt concentration measured wrt aqueous fraction.



solvated equivalent volume^{319,349}. For cation exchange resins of relatively large fixed site ions the selectivity order is:³¹⁹



However this is not a general trend and a cation exchanger with a small fixed ion can show a reversal of this selectivity order³¹⁹.

The nature of the anion will be unimportant unless ion-pairing of the solute and salt occurs. The salts used in the study were limited to those of high methanol solubility and were univalent unless indicated.

An estimate of the relative strengths of the ions used could not be based on retention times alone. The variation in methanol content of the mobile phase due to degassing had a significant effect on retention times. An internal standard of the effect of the salt was to take the point when the retention of the C_1 component equalled that of the C_{1a} component. This was termed the C_1/C_{1a} crossover point and is used in comparisons between solvents.

Plots of the retention characteristics of the components in mobile phases containing sodium iodide, lithium chloride and barium chloride are shown in Figures 5.7, 5.8, and 5.9 respectively. The crossover points estimated from graphs is given in Table 5.2 along with values found for other salts.

Figure 5.7. Effect of Sodium Iodide on Retention.

Conditions: As in Figure 5.6.

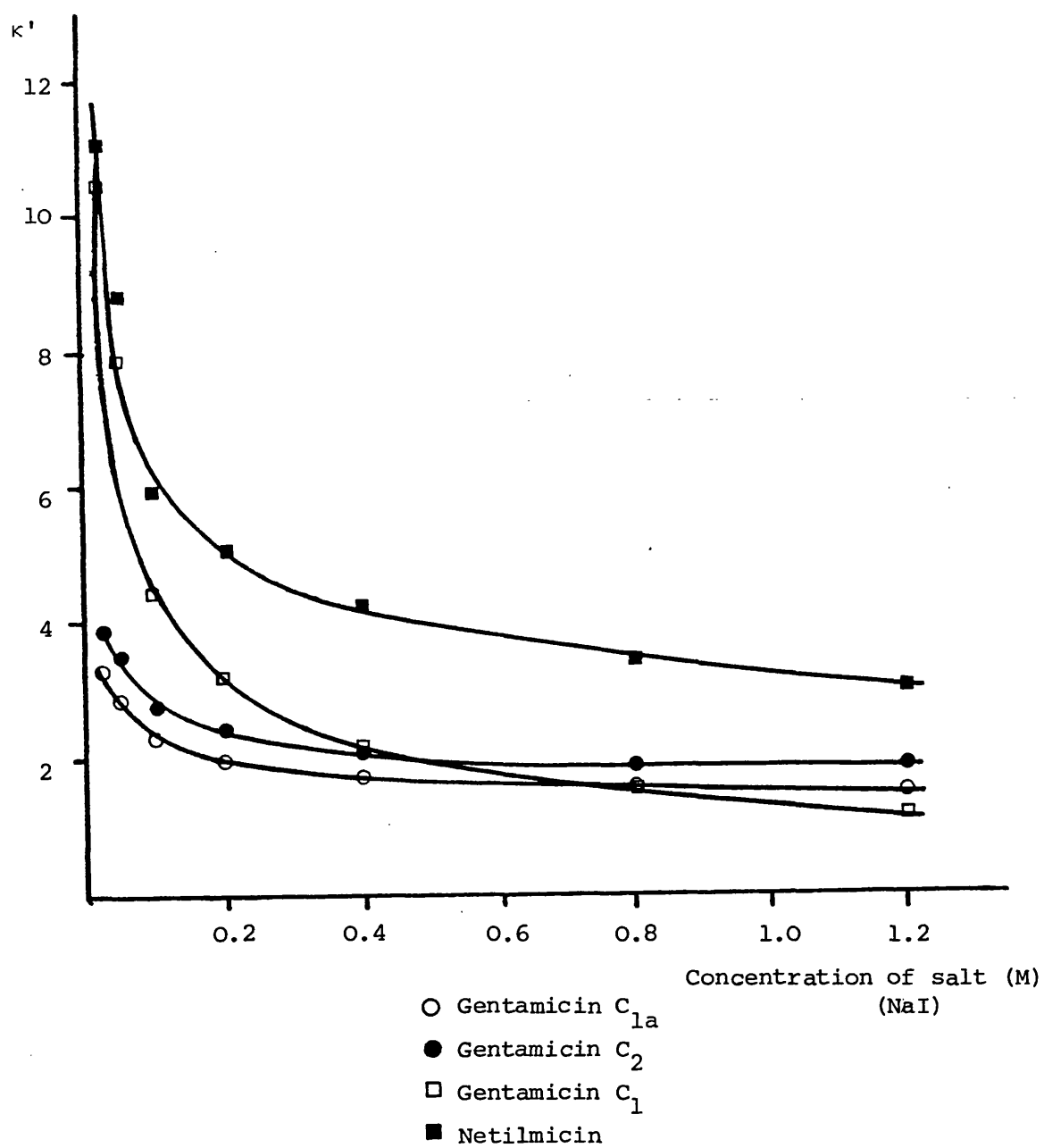


Figure 5.8. Effect of Lithium Chloride on Retention

Conditions : As in Figure 5.6

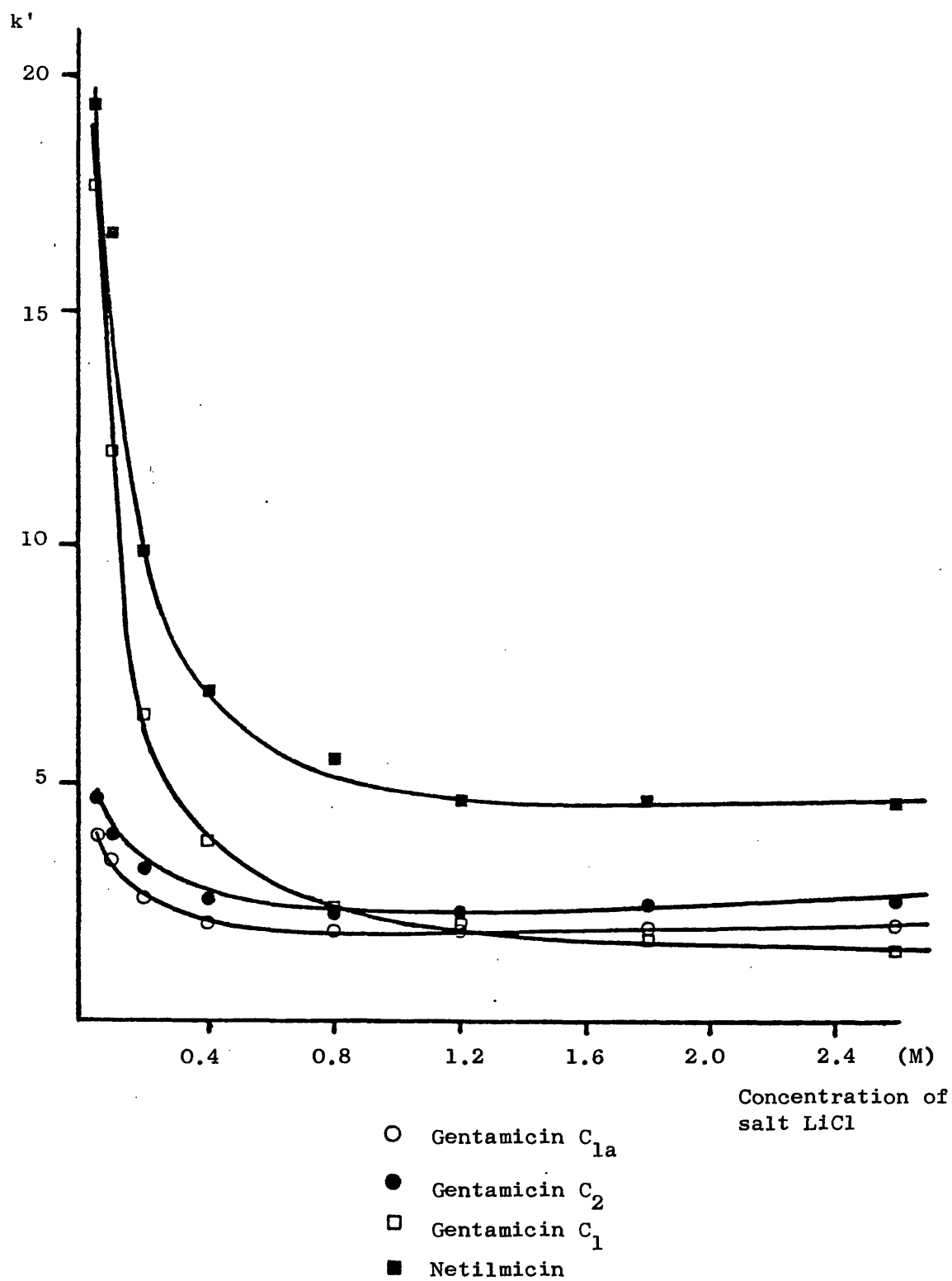


Figure 5.9. Effect of Barium Chloride on Retention

Conditions: As in Figure 5.6.

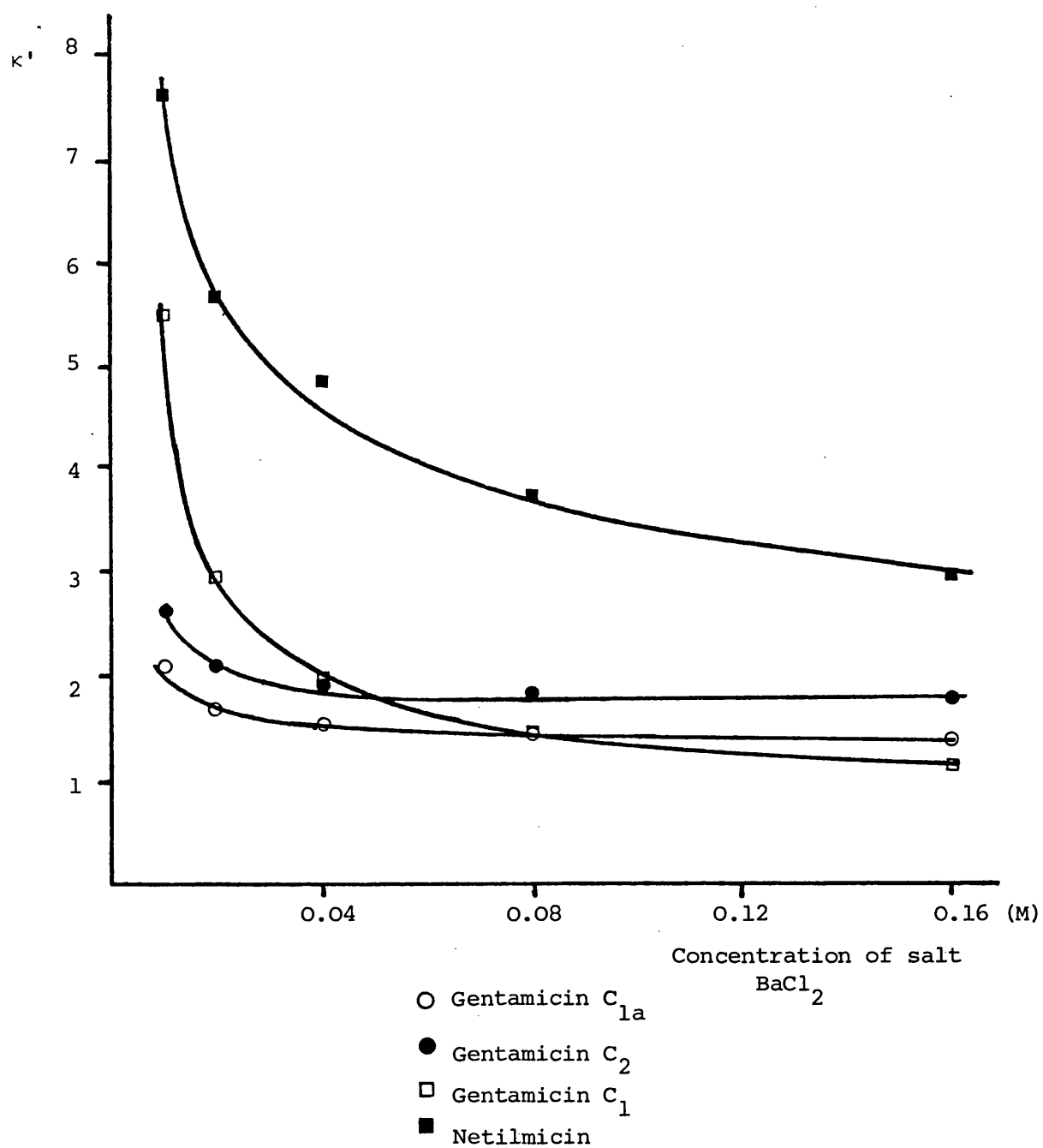


Table 5.2. Cross-over points of gentamicin C_1 and C_{1a} with different salts; observed and calculated

Column	Salt	Observed (M)	Calculated (M) log κ' vs. log $[x]$
Spherisorb	BaCl ₂	0.08	0.09
"	KI	0.45	0.37
"	KSCN	0.45	0.39
"	KNO ₃	-	0.52
"	NaI	0.80	0.68
"	NaNO ₃	0.80	0.78
"	LiCl	1.40	2.10
"	EDTA, K ₃ ⁺	-	0.56
Zorbax	KI	0.20	0.21
"	EDTA, K ₃	0.50	0.45
Hypersil	KI	0.004	0.004

The figures show that the components do behave in a similar fashion for all four cations, though as can be seen in Table 5.2 there is a significant difference in the respective eluting strengths. This is indicative that the column does differentiate between cations, which can only be explained by an ion-exchange mechanism. The concentrations given in Table 5.2 are the molar concentrations in the aqueous phase. However if the ionic strength contribution of barium is compared

to that of potassium the comparison of the cross-over point is of $I = 0.16$ to $I = 0.20$. This is perhaps a better comparison of the two ions of differing valency, although the barium ion is still stronger than the sodium and lithium ions. An interesting feature of Figure 5.8 is the rise in κ' value for the gentamicin C_{1a} and C_2 components with increasing salt concentration. This is as predicted by the solvophobic theory and reflects the concentration of salt exceeding 1.5 M (0.3 M mobile phase). The gentamicin C_1 and netilmicin curves do not show a similar increase perhaps reflecting a different mechanism.

The variation in column response to salt concentration was investigated, using column packings of Zorbax ODS and Hypersil ODS. The effect of potassium iodide concentration on the retention of the four compounds on Zorbax is shown in Figure 5.10. The column temperature had to be maintained at 44°C to produce satisfactory retention times and good peak shape. The graph is very similar to that found for the Spherisorb column (Figure 5.6) except that the intercept of the C_{1a} and C_1 lines is at a lower concentration of 0.2 M. The Zorbax ODS material is a monolayer coverage of ODS but with a high carbon loading of about 16% with no endcapping. It was thus expected to behave similarly to the Spherisorb column. The Hypersil column on the other hand has a monolayer coverage with a 9% carbon bonding but it is extensively endcapped with presumably few residual silanols present. This difference is reflected in Figure 5.11 which illustrates the effect of retention of potassium iodide on this column. All three components of gentamicin are eluted

Figure 5.10. Effect of Potassium Iodide on Retention on
a Zorbax Column

Conditions: Zorbax column; Mobile phase,
Methanol/Water 80:20, Salt measured w.r.t. aqueous

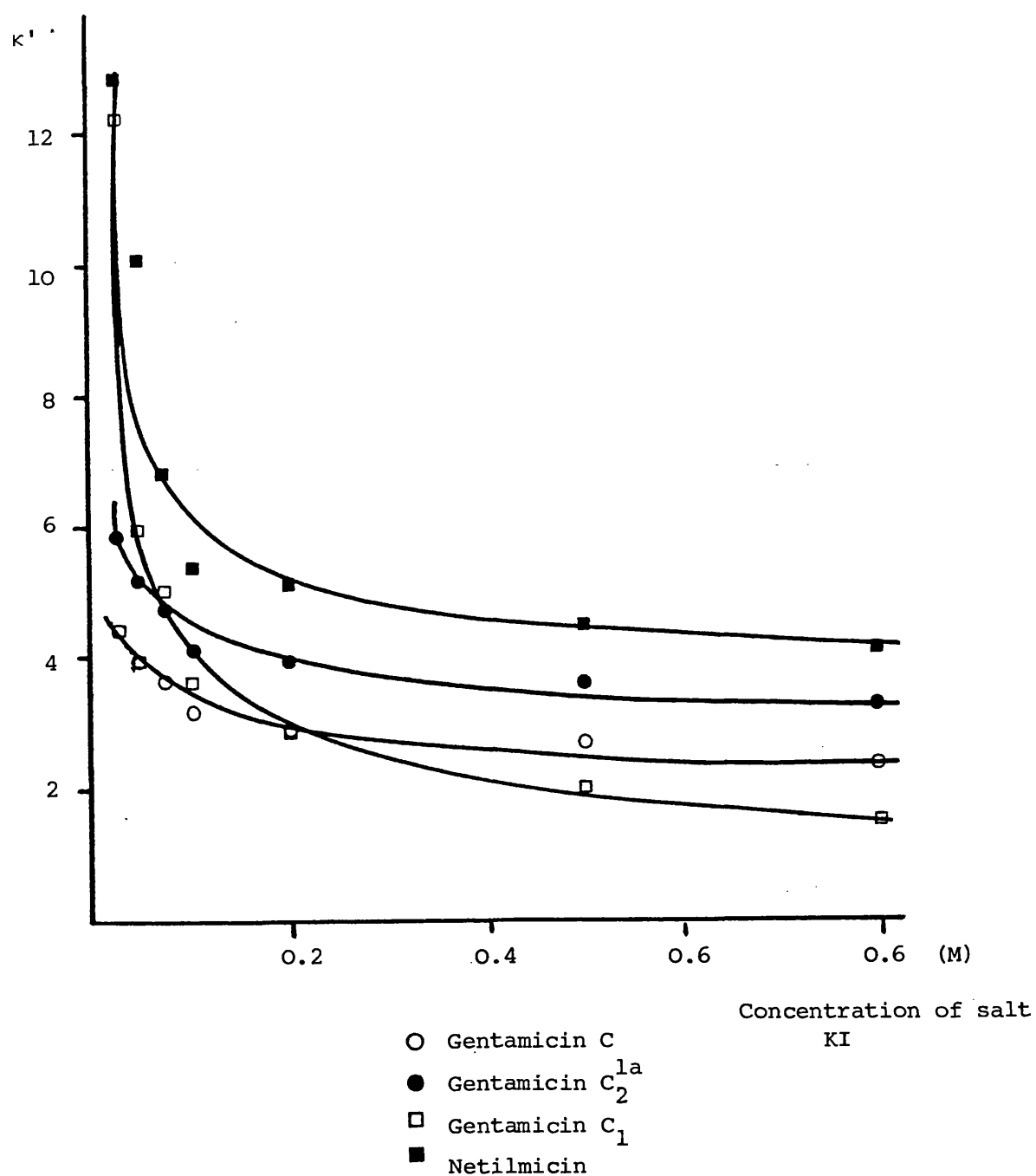
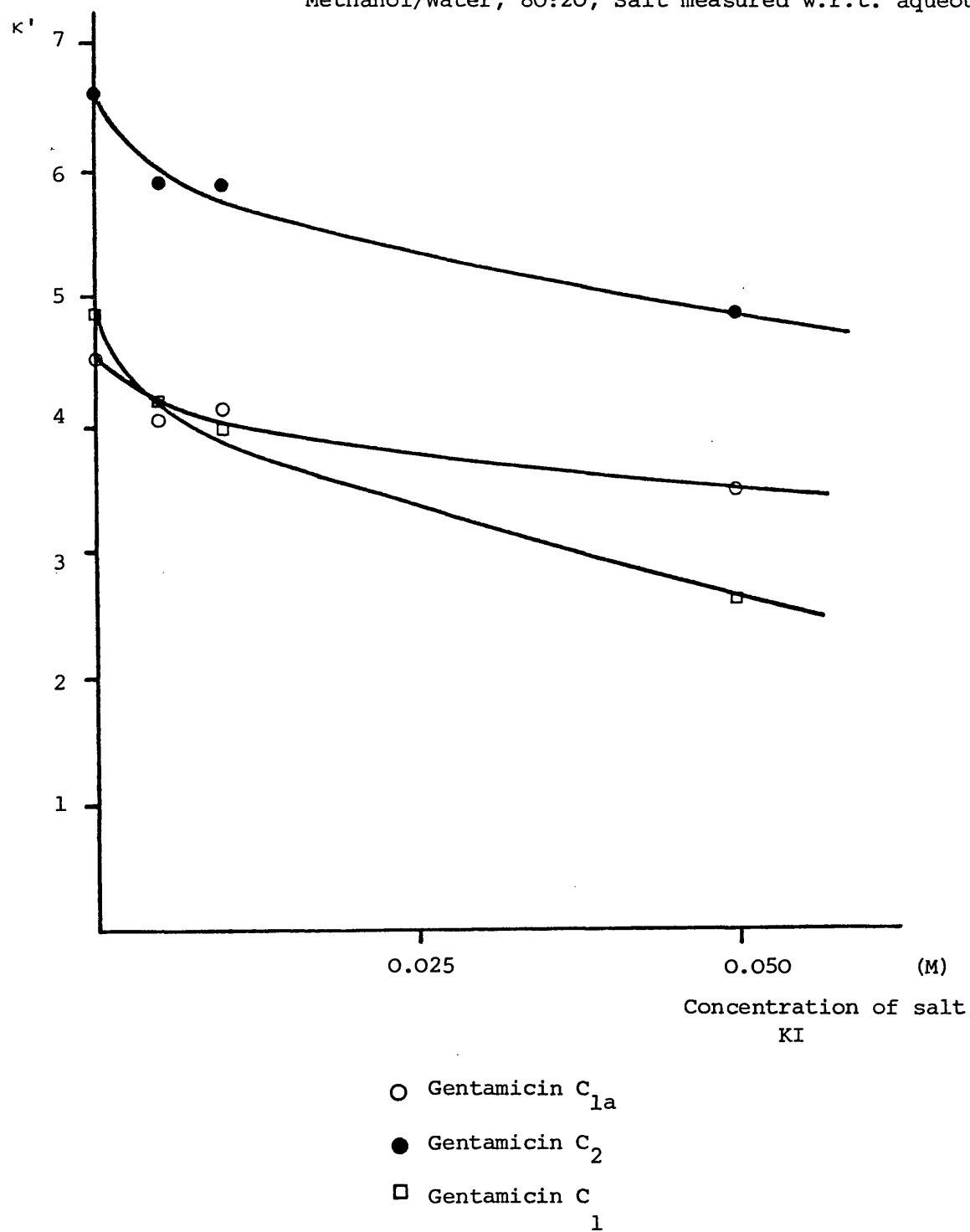


Figure 5.11. Effect of Potassium Iodide on Retention on a
Hypersil Column

Conditions: Hypersil column, Mobile Phase,
Methanol/Water, 80:20, Salt measured w.r.t. aqueous



in the absence of salt, which is different from the other two columns. Also a lower potassium iodide concentration is required to produce C_1/C_{1a} crossover and the gentamicin C_1 derivative is never retained for as long as on the two other columns. A comparison of retention on the three different columns with the same salt used indicates a common mechanism of retention operating, with the salt having an important role in determining the relative retention.

In published HPLC assays of gentamicin, ethylene-diaminetetra-acetic acid (EDTA), tripotassium salt is generally used in the elution solvent^{78,223}. Since the anion is more complex than the simple electrolytes used in Table 5.2 the effect of varying its concentration was examined. This is plotted in Figure 5.12 for the Spherisorb column and the concentration related to that of the potassium ion. The retention of the gentamicin C_{1a} and C_2 is longer than that observed in Figure 5.6 using potassium iodide. This may not be significant as it could be due to variation in the methanol content. However the difference in the retention of gentamicin C_1 between the two figures is large. There is a maximum value for κ' at about a concentration of 0.04 M which is not seen for any of the other salts. After this peak the κ' decreases at about the same rate as in Figure 5.6. The reason for this peak is unknown but may be due to the multivalent, large, organic anion which could be ion-pairing, or acting in an anomalous fashion at low concentration. The plot of the effect of EDTA, tri-potassium salt on retention

Figure 5.12. Effect of EDTA, K_3 on Retention on Spherisorb Column.

Conditions: As for Figure 5..6

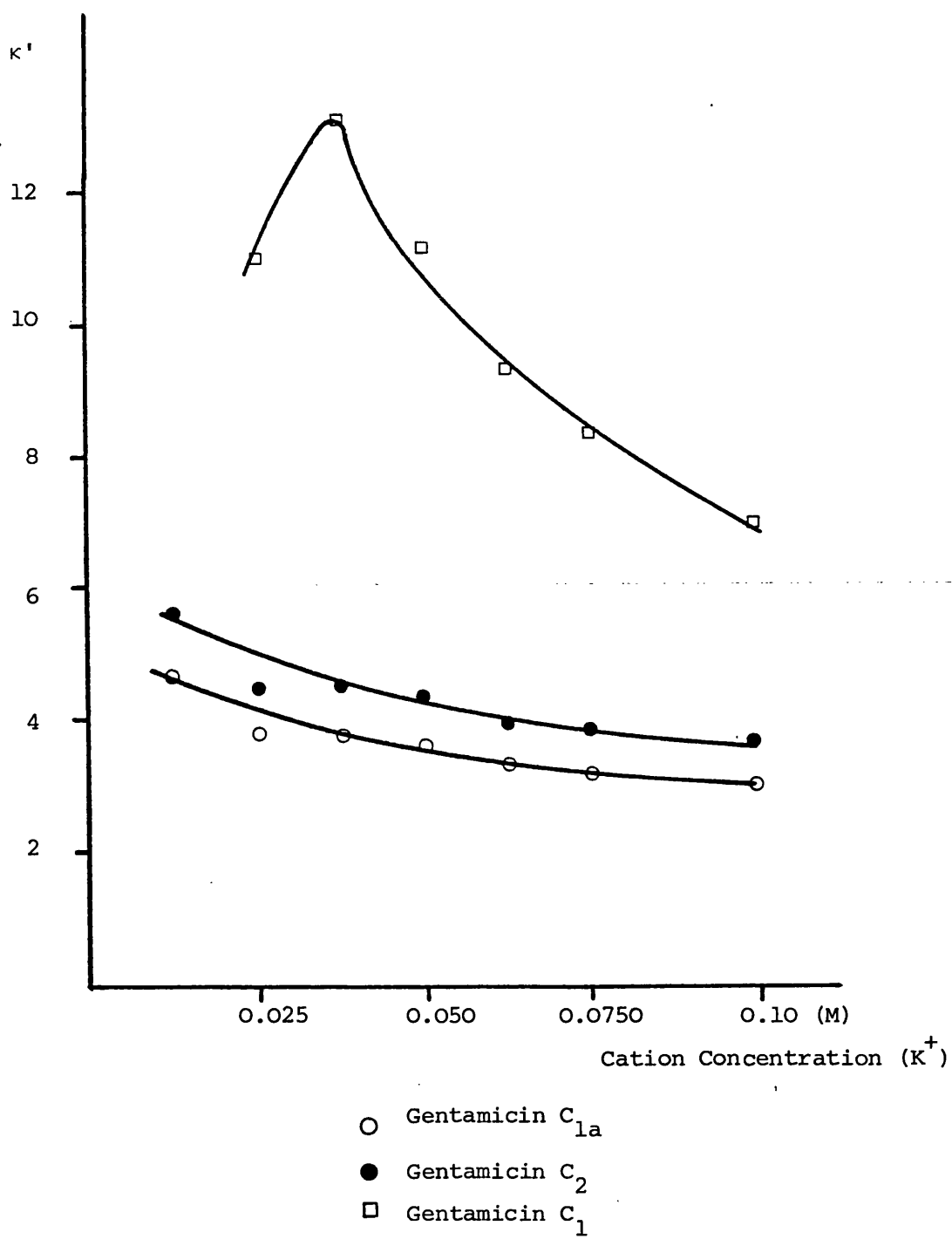
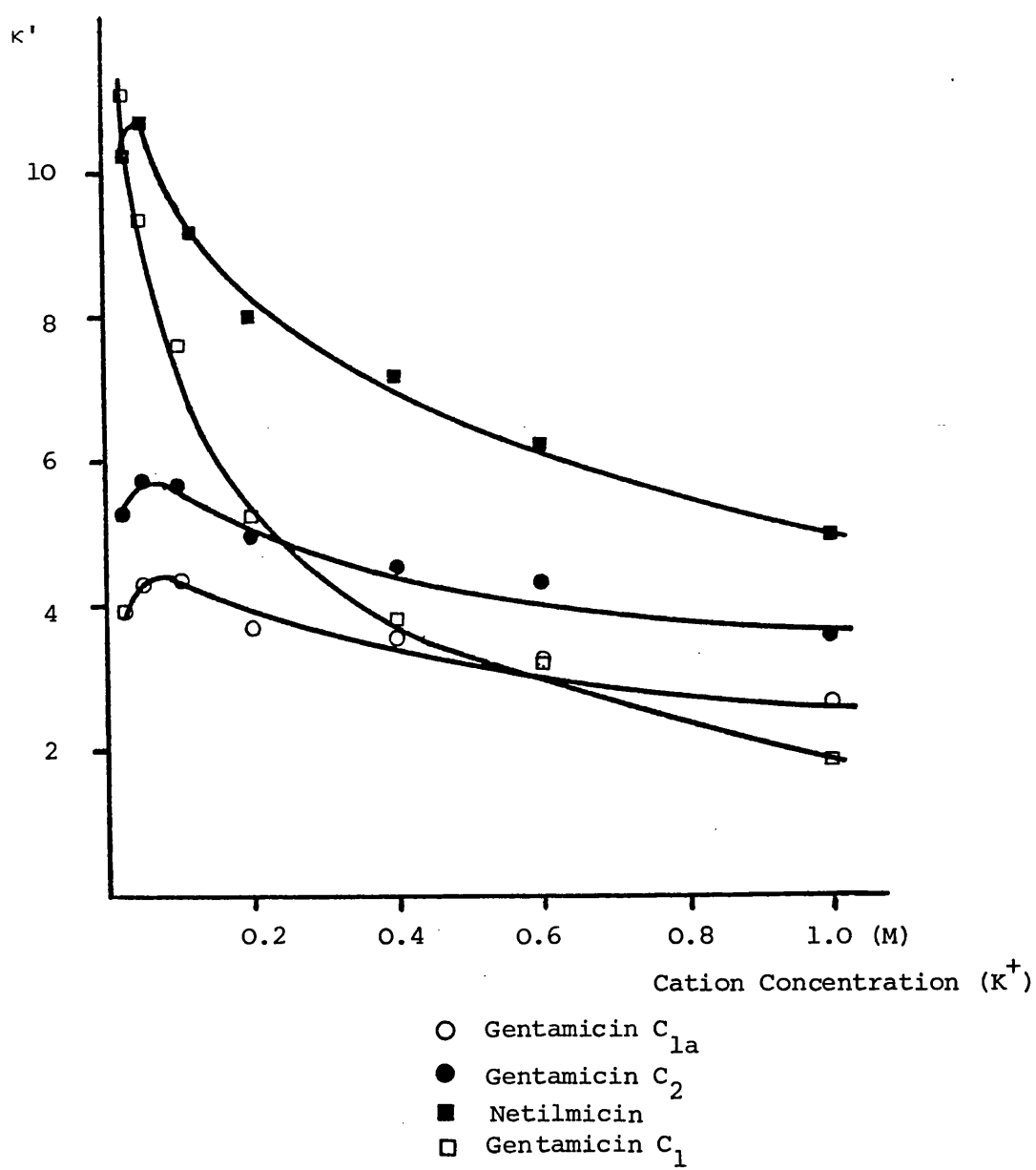


Figure 5.13. Effect of EDTA, K_3 on Retention on Zorbax

Column

Conditions: As in Figure 5.10.



using a Zorbax column is shown in Figure 5.13. This time it is all the other components except gentamicin C_1 which experience a maximum κ' value at low salt concentrations (0.05 M). No plausible reason can be suggested for the effect or why only some components experience this effect but not consistently. The cross-over point of the C_1/C_{1a} is at 0.5 M indicating that the EDTA salt is less effective than the simple electrolyte. This may arise from a low dissociation constant for the salt. The EDTA salt on both columns has been shown to exhibit the same pattern of behaviour and a larger effective concentration of potassium was required to produce similar retention to the simple electrolytes.

If an ion-exchange process is a major determinant of retention it would be useful to quantitate the amount of silanol groups present in each stationary phase. Qualitative tests for silanols were mentioned in the introduction, but a semi-empirical estimate of silanol groups present can be made from the surface coverage of the ODS material. This obviously is limited and cannot take into account coverage by endcapping reagents. It is assumed that the total amount of residual silanols available for reaction is $4.5 \mu\text{moles m}^{-2}$, and that the figures taken for surface coverage from manufacturers data are correct. The coverage of residual silanols is therefore:

Table 5.3. Percentage coverage of silanols on different columns

	Surface coverage ($\mu\text{mol m}^{-2}$)	% coverage (silanols)
Spherisorb	1.36	30
Zorbax	2.50	54
Hypersil	3.52	77

The figures are only intended as a rough guide to polarity of the phases and not as accurate determinations. Spherisorb probably has a greater coverage of silanols than shown because a partial end-capping reaction is performed. However, the effect of the silanols is likely to be greater than the Zorbax material because of the lower carbon loading shielding the groups. Hypersil would have a much smaller influence of silanols and this is supported by Figure 5.11. The above order of values is consistent with the need for lower concentration of potassium iodide with Zorbax and especially Hypersil when compared to Spherisorb.

For ion-exchange the capacity factor (κ') is usually directly proportional to the reciprocal of the concentration of the metal ion for a univalent solute ion and proportional to the reciprocal of the square of the concentration of the metal ion for a divalent solute²⁴¹. The calculated slopes of κ' against $\frac{1}{\text{conc}^2}$, $\frac{1}{\text{conc}}$ and $\frac{1}{\sqrt{\text{conc}}}$ for gentamicin C_1 in a potassium iodide mobile phase are shown in Table 5.4.

Table 5.4. Slopes of possible ion-exchange relationships for potassium iodide

	$\kappa' \text{ v's } \frac{1}{\text{conc}^2}$	$\kappa' \text{ v's } \frac{1}{\text{conc}}$	$\kappa' \text{ v's } \frac{1}{\sqrt{\text{conc}}}$
r	0.958	0.988	0.995
slope	0.00134	0.148	1.75

A plot of $\kappa' \text{ v's } \frac{1}{\sqrt{\text{conc}}}$ for all compounds in the potassium iodide mobile phase on Spherisorb is shown in Figure 5.14. For other salts the same relative correlations are found for gentamicin C_1 with the $\frac{1}{\sqrt{\text{conc}}}$ relationship giving the most linear fit. This was used because the κ' against $\frac{1}{\text{conc}}$ graph gave a curve and it was apparent that $\frac{1}{\sqrt{\text{conc}}}$ would decrease this tendency, though not entirely eliminate it. The other components also show the highest correlation with κ' against $\frac{1}{\sqrt{\text{conc}}}$, but they are much less significant. The reason why κ' against $\frac{1}{\text{conc}}$ is not linear may arise from the retention not being solely governed by ion-exchange.

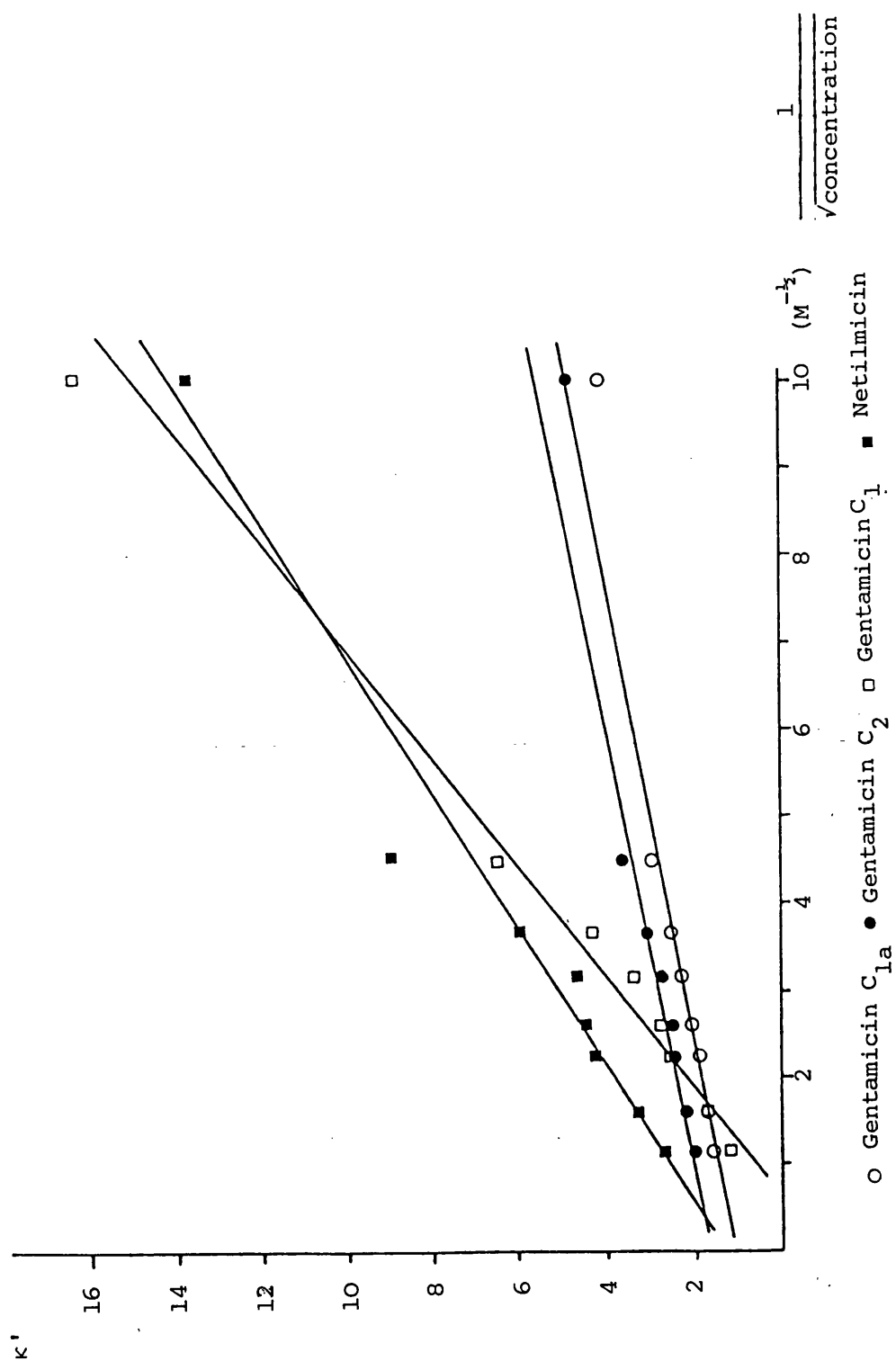
In ion-exchange the logarithm of the distribution coefficient (K_A) is related to the logarithm of the salt concentration^{319,350}. The distribution coefficient can be related to κ' by:

$$\kappa' = K_A \frac{V_s}{V_m}$$

V_s is volume of stationary phase

V_m is volume of mobile phase

Figure 5.14. Plot of κ' against the reciprocal of the square root of $[\text{salt}]$, (potassium iodide)



The two volumes are assumed to be constant for a given column and thus $\ln \kappa'$ against $\ln [\text{salt}]$ should be linear. However a general adsorption isotherm, the Freundlich is denoted by:

$$\kappa' = a [\text{salt}]^{-b}$$

$$\ln \kappa' = \ln a - b \ln [\text{salt}]$$

Greving et al.³³⁶ used this adsorption isotherm to explain the effect of differing pairing ions on ion-pair adsorption of quaternary ammonium compounds, using silica as the stationary phase. Bromide and chlorate ions produced different retention values and ion-exchange was excluded because the nature of the cations did not influence retention behaviour. However it serves to illustrate that adherence to this relationship is not solely a property of ion-exchange and the equation could describe many types of adsorption. Using this equation for the gentamicin C components and netilmicin, the parameters a and b were calculated for the various salts and columns. The results are given in Tables 5.5 and 5.6 and plots of some gentamicin C curves in different mobile phases, and all the components in the same sodium iodide mobile phase are given in Figures 5.15 and 5.16. The gentamicin C_1 plots showed the most significant relationship with the highest correlation (r). The ' a ' quantity is the exponential intercept and b is the slope of the graph. The slope of the line is an indication of the strength of interaction of the salt and compound, whilst the intercept is a measure of the

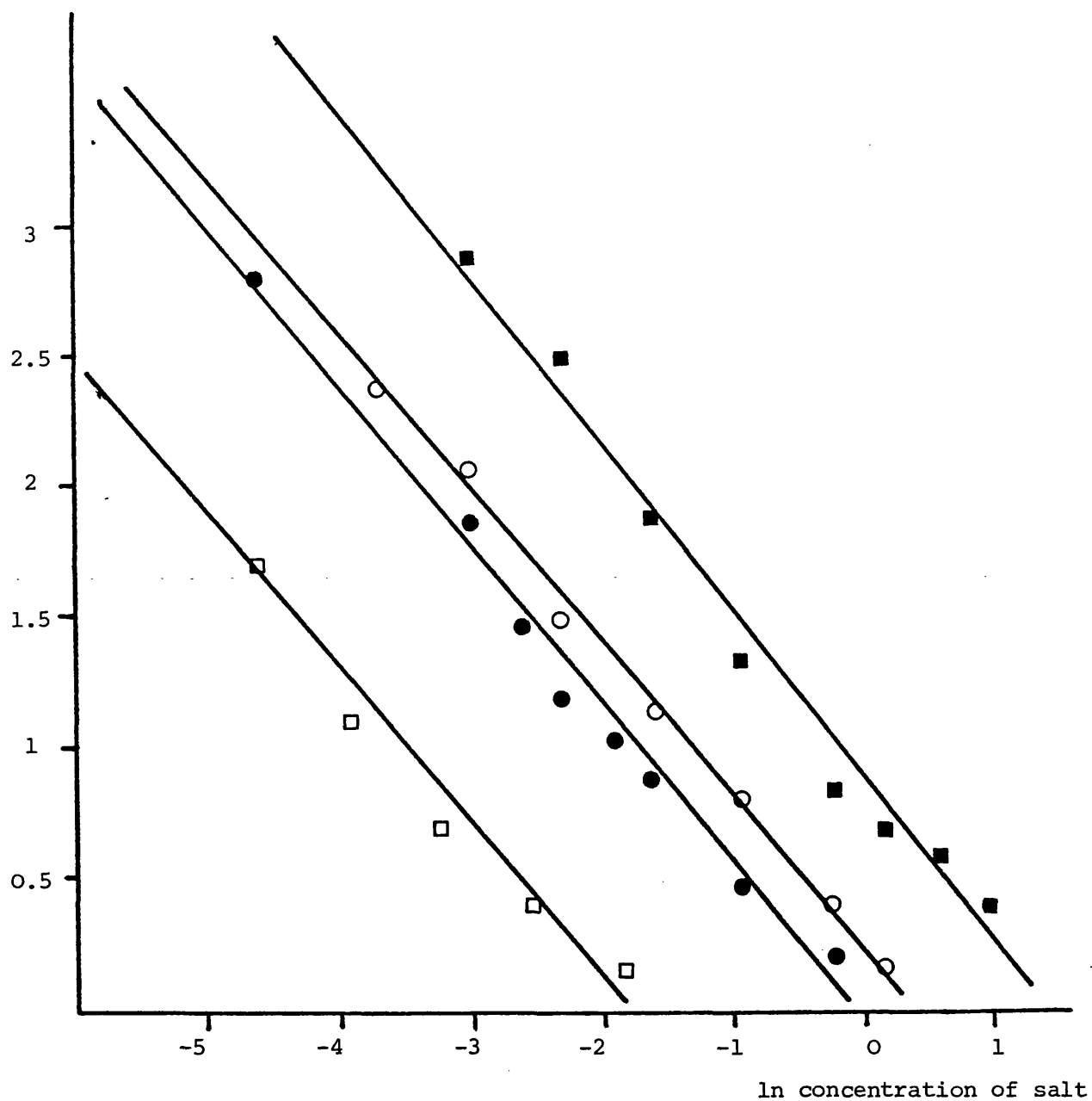
Table 5.5. Gentamicin C_{1a} and C_2 calculated for various mobile phases ($\ln \kappa'$ v's $\ln [\text{salt}]$)

Column	Salt	a	b	r
<u>Gentamicin C_{1a}</u>				
Spherisorb	KI	1.34	-0.24	0.985
"	KSCN	1.35	-0.29	0.959
"	KNO ₃	1.29	-0.32	0.996
"	NaI	1.49	-0.20	0.986
"	NaNO ₃	1.64	-0.21	0.981
"	LiCl	1.76	-0.25	0.982
"	BaCl ₂	1.04	-0.13	0.939
"	EDTA, K ₃	2.08	-0.17	0.940
Zorbax	KI	2.34	-0.16	0.968
"	EDTA, K ₃	2.92	-0.15	0.936
Hypersil	KI	2.69	-0.09	0.965
<u>Gentamicin C_2</u>				
Spherisorb	KI	1.73	-0.22	0.987
"	KSCN	1.70	-0.28	0.969
"	KNO ₃	1.61	-0.31	0.994
"	NaI	2.00	-0.17	0.967
"	NaNO ₃	2.28	-0.11	0.962
"	LiCl	2.16	-0.24	0.985
"	BaCl ₂	1.37	-0.12	0.928
"	EDTA, K ₃	2.34	-0.19	0.965
Zorbax	KI	3.16	-0.15	0.964
"	EDTA, K ₃	3.86	-0.15	0.960
Hypersil	KI	3.78	-0.09	0.967

Table 5.6. Gentamicin C_1 and netilmicin, calculated for various mobile phases ($\ln \kappa'$ v's $\ln[\text{salt}]$)

Column	Salt	a	b	r
<u>Gentamicin C_1</u>				
Spherisorb	KI	0.94	-0.60	0.992
"	KSCN	0.90	-0.72	0.986
"	KNO_3	0.99	-0.73	0.996
"	NaI	1.29	-0.57	0.998
"	NaNO_3	1.48	-0.62	0.995
"	LiCl	2.37	-0.65	0.991
"	BaCl_2	0.38	-0.55	0.981
"	EDTA, K_3	1.57	-0.65	0.998
Zorbax	KI	1.35	-0.51	0.976
"	EDTA, K_3	2.18	-0.52	0.984
Hypersil	KI	1.48	-0.20	0.960
<u>Netilmicin</u>				
Spherisorb	KI	2.24	-0.39	0.978
"	KSCN	2.10	-0.56	0.952
"	KNO_3	2.22	-0.47	0.991
"	NaI	3.13	-0.33	0.992
"	NaNO_3	3.13	-0.42	0.984
"	LiCl	4.87	-0.48	0.991
"	BaCl_2	1.61	-0.33	0.997
Zorbax	KI	3.42	-0.31	0.919
"	EDTA, K_3	5.37	-0.25	0.983

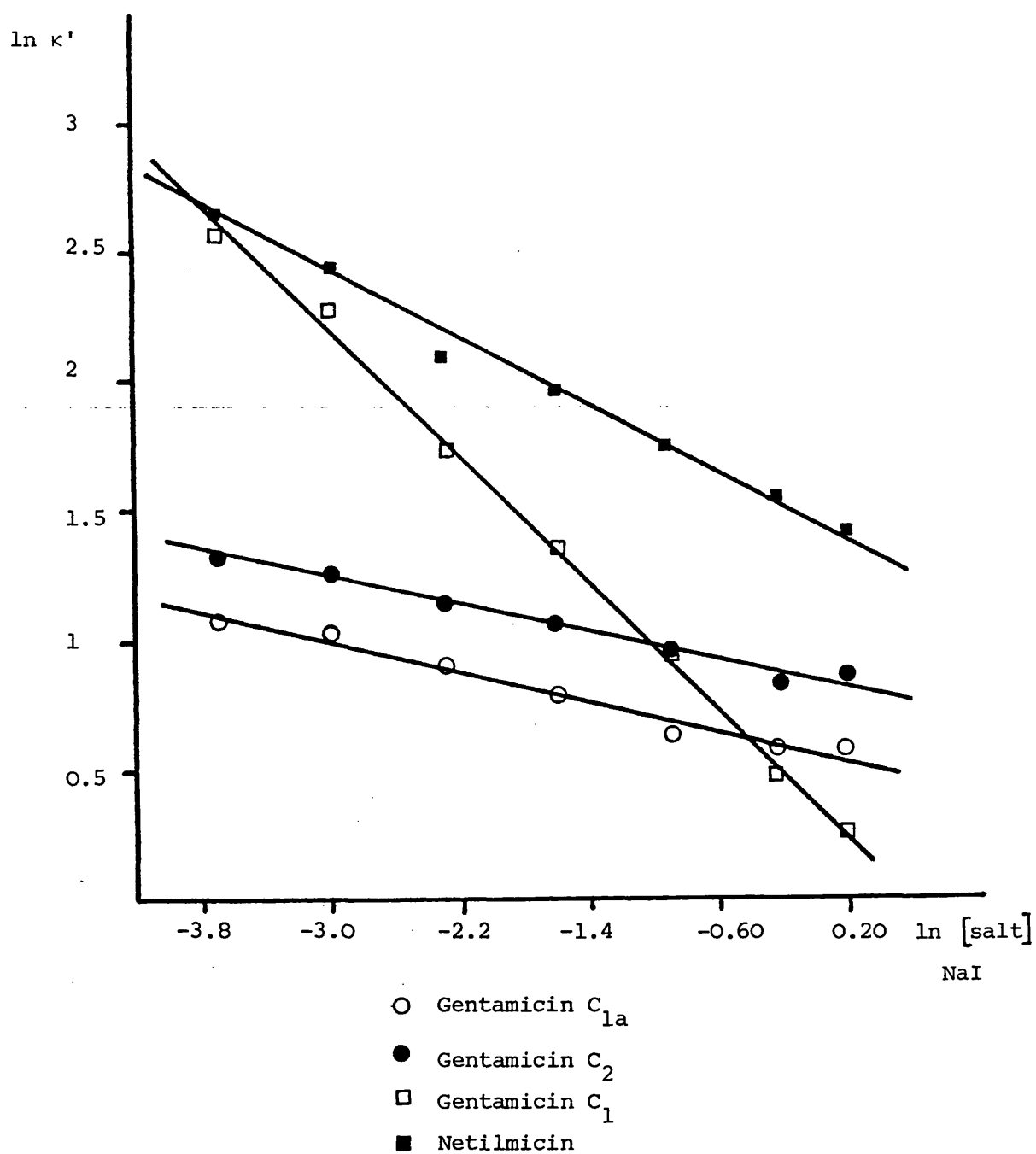
Figure 5.15. Plot of $\ln \kappa'$ against \ln concentration of salt for gentamicin C_1 in various mobile phases on a Spherisorb column



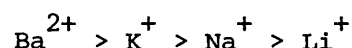
- LiCl
- BaCl₂
- KI
- NaI

Figure 5.16. Plot of $\ln \kappa'$ against \ln concentration of salt for all components in sodium iodide mobile phase

Conditions: As in Figure 5.7



κ' value at unit concentration. The scatter of b values for one compound in the different mobile phases indicates that b is a measure of the general adsorption of the solute and is not a measure of specific interactions arising from the nature of the cation. The b value on the Hypersil column is smaller, but the Zorbax column yields similar b values to the Spherisorb column. The b value for gentamicin C_1 is greater than for any other component, whilst the C_{1a} and C_2 components are nearly identical, showing the same adsorption pattern. The a values show the relative retention at $[\text{salt}] = 1.00$ and reveal the expected selectivity trend of the cation exchange:



The EDTA, tri potassium salt shows longer retention than the other potassium salts which, as previously mentioned, is probably an anion effect.

These results suggest that at least the gentamicin C_1 component may be retained by an ion-exchange mechanism with the other components having other factors, and possibly another mechanism, involved. It thus also confirms the non-linear plot of the temperature dependence of retention (Figure 5.2).

Various authors have reported a two site adsorption retention model on reverse phase packings^{327,330,331} and equations have been formulated to express the unusual retention. Two

of these models were tested on the data obtained in this study in an attempt to gain further insight into the effect of salt on retention of the gentamicin components.

Stranahan³⁵¹ developed a four parameter thermodynamic model to account for the effect of salts in reversed phase chromatography with special reference to ion-pair formation. The distribution coefficient (K) of a solute was related to the concentration of an "ion-interaction reagent" (IIR) with regard to various physical properties of the solute and system. The action of the IIR was described as comprising of ion-interaction and interfacial tension effects. In applying this equation to the present set of data it was decided to disregard the interfacial tension effect, as the simple electrolytes used here would influence the tension less than the surfactants used in the paper. It also simplified calculation as the equation was reduced to three unknown parameters. The equation devised by Stranahan was:

$$K = \exp \left(\ln \frac{V_1^0}{V_a^0} + \ln \gamma_{il} + \frac{(\delta - \delta_i^0) S_i}{RT} - \frac{w_{ij} X_{JA}}{RT} \right)$$

where V_1^0 is average molar volume of solute in liquid phase

V_a^0 is average molar volume of solute in adsorbed phase

γ_{il} is activity coefficient of solute in liquid phase

δ is interfacial tension of system

δ_i^0 is interfacial tension of pure solute in equilibrium

with solid adsorbent

S_i is area on surface of adsorbent of 1 mole of solute

w_{ij} is energy of interaction in adsorbed phase of solute

and IIR

x_{JA} is mole fraction of IIR in adsorbed phase

which can be expressed as:

$$\kappa' = \exp \left\{ \beta_0 + \beta_1 \left[\frac{C}{C + \beta_2} \right] \right\}$$

C is concentration of salt (IIR) in mobile phase

β_0 is constant of first three terms

β_1 is constant related to interaction energy of solute and salt (IIR)

β_2 is constant related to energy of adsorption of IIR.

The determination of the three parameters in the equation was achieved by rearranging the equation to give:

$$\frac{1}{(\ln \kappa' - \beta_0)} = \frac{1}{\beta_1} + \frac{\beta_2}{\beta_1} \cdot \frac{1}{C}$$

To calculate the best fitting straight line the parameter, β_0 , was estimated for the potassium iodide mobile phase on the Spherisorb column using the y-intercept of a plot of $\frac{1}{\kappa'}$, against concentration for the first three points. The value of β_0 which gave the highest correlation coefficient for the equation, using the potassium iodide salt, was used to calculate

the other parameters. The logarithm of the capacity factor at zero salt concentration is equivalent to β_0 , so this could be used for the Hypersil column. For the individual components on the Spherisorb column the same β_0 value was used wherever possible and separate optimum values were found for the Zorbax column. The values of the three parameters for all salts and columns are given in Tables 5.7 and 5.8, as are the Gibbs free energy of adsorption (ΔG°) calculated from:³⁵¹

$$\Delta G^\circ = RT \ln \frac{\beta_2}{277.5}$$

The plot of the three parameter equation applied to the retention in the potassium iodide mobile phase is shown in Figure 5.17, and applied to the ordinary κ' against concentration plot in Figure 5.18. The latter shows that fitting the parameters can reproduce the κ' values fairly well though the flattening out of the netilmicin curve is not accounted for.

The term β_2 is a measure of the interaction of the salt (IIR) with the stationary phase, which is most likely to occur at the silanol groups. The ΔG° value calculated should ideally be the same for all solutes in the mobile phase since it is independent of solute. The larger the ΔG° for the salt the less concentration should be required to elute the solutes. The average ΔG° taken from all four compounds for all the salts is given in Table 5.9. The pattern of the figures in Table 5.9 is not entirely consistent with experimental observations, since the sodium salts possess a larger ΔG° value than the

Table 5.7. Stranahan's equation applied to gentamicin C_{1a} and C_2 curves

Column	Salt	β_0	β_1	β_2	r	ΔG° (KJ mol ⁻¹)
<u>Gentamicin C_{1a}</u>						
Spherisorb	KI	1.53	-1.29	0.09	0.999	-20.24
"	KSCN	"	-2.17	0.35	0.971	-16.82
"	KNO ₃	"	-1.50	0.19	0.999	-18.36
"	NaI	"	-1.08	0.05	0.985	-21.72
"	NaNO ₃	"	-1.10	0.10	0.999	-19.97
"	LiCl	"	-1.12	0.22	0.994	-17.99
"	BaCl ₂	"	-1.25	0.006	0.993	-27.06
"	EDTA K ₃	"	-0.56	0.20	0.888	-18.23
Zorbax	KI	1.50	-1.18	0.36	0.955	-17.52
"	EDTA, K ₃	"	-0.91	5.34	0.940	(-10.41)
Hypersil	KI	1.51	-0.23	0.01	0.890	-25.77
<u>Gentamicin C_2</u>						
Spherisorb	KI	1.67	-1.29	0.11	0.999	-19.73
"	KSCN	"	-2.28	0.44	0.970	-16.24
"	KNO ₃	"	-1.74	0.28	0.992	-17.38
"	NaI	"	-1.00	0.06	0.993	-21.26
"	NaNO ₃	"	-1.02	0.12	0.998	-19.51
"	LiCl	"	-3.18	1.04	0.939	-14.07
"	BaCl ₂	"	-1.14	0.005	0.986	-27.52
"	EDTA, K ₃	"	-0.90	0.08	0.974	-20.53
Zorbax	KI	1.97	-0.86	0.08	0.993	-21.48
"	EDTA K ₃	1.79	-0.72	3.04	0.968	(-11.90)
Hypersil	KI	1.88	-0.27	0.009	0.886	-26.04

Table 5.8. Stranahan's equation applied to gentamicin C_1 and netilmicin curves

Column ;	Salt	β_0	β_1	β_2	r	ΔG° (KJ mol ⁻¹)
<u>Gentamicin C_1</u>						
Spherisorb	KI	3.22	-3.21	0.06	0.999	-21.26
"	KSCN	"	-3.76	0.13	0.995	-19.31
"	KNO ₃	"	-3.23	0.11	0.999	-19.73
"	NaI	"	-2.83	0.06	0.990	-21.26
"	NaNO ₃	"	-2.85	0.10	0.995	-19.97
"	LiCl	"	-4.20	0.54	0.994	-15.72
"	BaCl ₂	"	-3.28	0.01	0.999	-25.77
"	EDTA, K ₃	"	-3.32	0.62	0.998	-15.38
Zorbax	KI	3.22	-3.03	0.07	0.987	-21.84
"	EDTA, K ₃	"	-2.03	0.16	0.948	-19.66
Hypersil	KI	1.59	-0.53	0.01	0.858	-25.77
<u>Netilmicin</u>						
Spherisorb	KI	2.77	-2.50	0.15	0.998	-18.95
"	KSCN	3.55	-3.01	0.10	0.991	-19.97
"	KNO ₃	2.77	-1.92	0.12	0.991	-19.51
"	NaI	"	-1.73	0.09	0.998	-20.24
"	NaNO ₃	"	-2.01	0.22	0.996	-17.99
"	LiCl	"	-1.62	0.45	0.989	-16.18
"	BaCl ₂	"	-1.70	0.01	0.993	-25.77
Zorbax	KI	2.77	-3.37	0.34	0.972	-17.67
"	EDTA, K ₃	"	-1.04	0.34	0.978	-17.67

Figure 5.17. Plot of form of Stranahan's Equation to determine parameters.

Conditions: Taken from Figure 5.6.

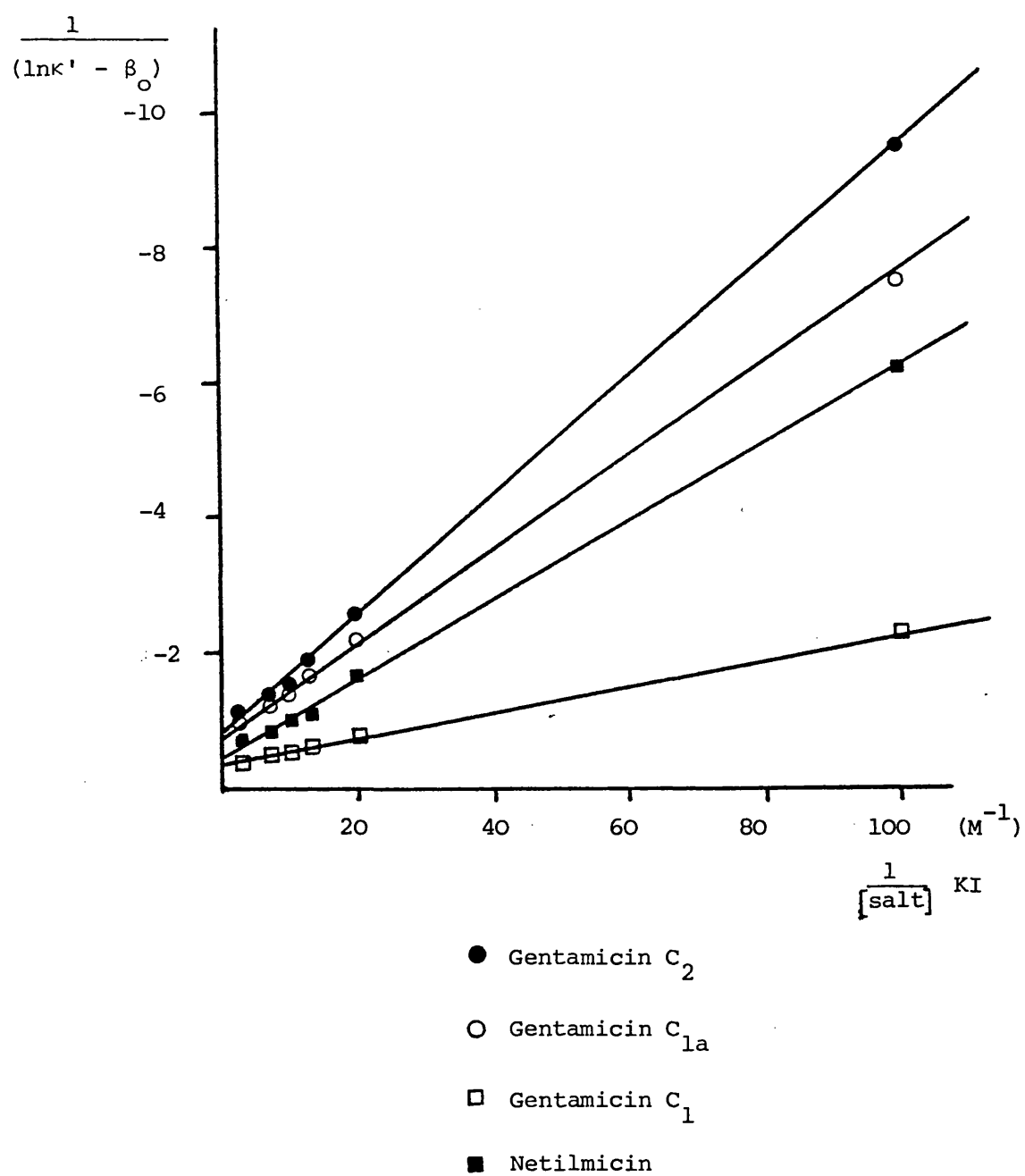
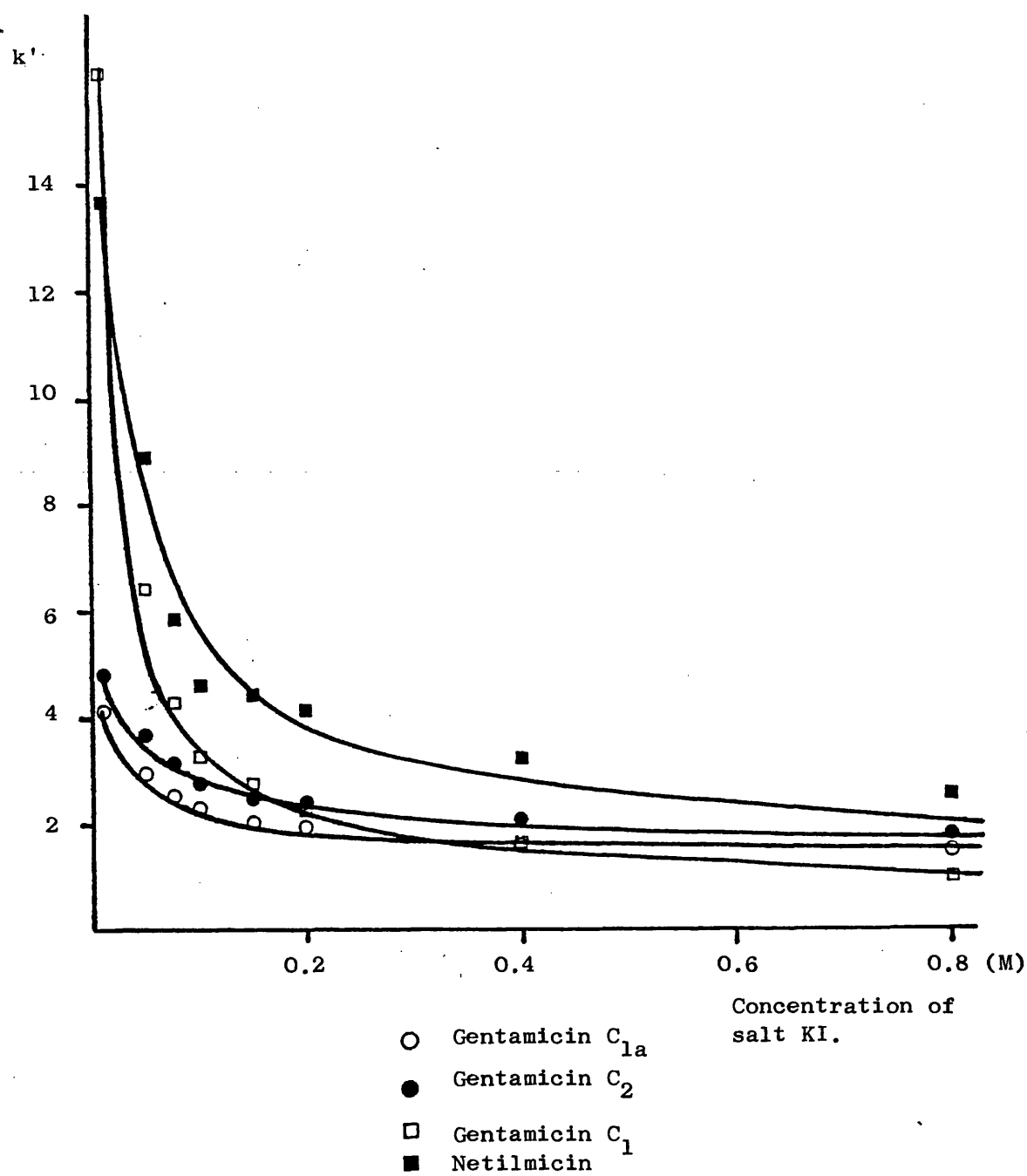


Figure 5.18. Plot of Theoretical Curves from Stranahan's Equation to points found in Experiment for Potassium Iodide.

Conditions : As in Figure 5.6



potassium salts although the difference is small. The barium and lithium salts behave as expected whilst the EDTA salt is close to the value for other potassium salts although it is subject to large variations. The value of the potassium ion

Table 5.9. Free energy of adsorption of the salts

Column	Salt	ΔG° (KJ mol ⁻¹)	Standard Deviation
Spherisorb	KI	-20.04	0.97
"	KSCN	-18.09	1.83
"	KNO ₃	-18.75	1.09
"	NaI	-21.12	0.63
"	NaNO ₃	-19.36	0.94
"	LiCl	-15.99	1.61
"	BaCl ₂	-26.53	0.90
"	EDTA, K ₃	-18.05	2.58
Zorbax	KI	-19.63	2.35
"	EDTA, K ₃	-18.67	-
Hypersil	KI	-25.86	0.16

on the Hypersil column is significantly larger than for the other two columns and may indicate that this is not a true measure of the ΔG° term. This is probably because the ions are not following surfactant behaviour, which is a premiss of the equation for ΔG° . Stranahan³⁵¹ determined a value of -8.62 KJ mol⁻¹ for octylamine and -4.98 to -16.95 KJ mol⁻¹

for a series of anionic surfactants. These were most probably being adsorbed by hydrophobic bonding since there was little change in ΔG° between cationic and anionic species. The higher values of ΔG° reported here, may reflect the greater energies of ionic bonding, though comparison with Stranahan's results cannot be made with confidence.

The β_1 term is related to the interaction energy of the salt and solute, with the negative sign indicating a repulsive effect. The size of the repulsion is greatest for the gentamicin C_1 solute, with the netilmicin also larger than the gentamicin C_{1a} or C_2 derivative values. This is possibly due to the presence of two secondary amino groups on the gentamicin C_1 and netilmicin, or to the greater basicity of the additional secondary amino group. The size of this interaction is variable and is also different on the individual columns.

A fairly good fit of the data is obtained by this method despite the IIR used being salts and not surfactants. The constraint of using the same β_0 value reduced the correlation since there was some variation in the mobile phase composition in the absence of salt. The thermodynamic quantities obtained with the equation may not be completely trustworthy for the reasons mentioned and also because of the omission of the surface tension term. It does demonstrate that simple electrolytes can be compared with surfactants, at least in some applications of BPC.

Organic amines have been used in reversed phase BPC because they are thought to mask silanol groups which could influence retention. The theory of dual site adsorption has produced equations which directly relate the capacity factors of solute to the concentration of amine used^{327,330}. The masking of the silanols, by such amines as dimethyloctylamine and hexadecyltrimethylammonium bromide, is thought to increase the regularity of reverse phase characteristics. Bij et al.³³⁰ derived an equation, assuming that there are two binding sites of different affinity available, which are probably the hydrocarbon ligand and the silanol groups. However since similar behaviour can be observed on silica^{330,344,345}, the equation has to be considered as a general expression of reverse phase chromatography. The simple equation derived is:

$$\frac{[A]}{\kappa'_0 - \kappa'} = \frac{1}{\kappa_2 K_A} + \frac{[A]}{\kappa_2}$$

where $[A]$ is concentration of masking agent

κ'_0 is capacity factor in absence of agent

K_A is binding constant of agent to silanol groups

κ_2 is capacity factor due to silanophilic binding in absence of agent.

Although this equation was devised to account for different sites of adsorption, where the silanols are thought to adsorb by hydrogen bonding or electrostatic interactions, it can also be applied to the ion-exchange/hydrophobic adsorption thought

to be occurring here. The salt concentrations required would be assumed to be greater than those required by the amine because of the hydrophobic environment of the silanol site. Since only the Hypersil column could elute peaks at zero concentration of salt the κ'_0 values for the other two columns were taken as the exponential (β_0) values from the Stranaham equation. Using these figures the plot of $\frac{[A]}{\kappa'_0 - \kappa'}$ against $[A]$ was made for all compounds and salts, with an example shown in Figure 5.19 of the solutes in potassium iodide mobile phase on the Spherisorb column. The κ_2 , K_A and C_A^{*30} values were calculated for each salt and column as shown in Tables 5.10 and 5.11. Here C_A^* represents the concentration of salt required to mask 90% of the silanols present and is useful as a guide to the effectiveness of the salt. This was calculated from $10 K_A^{-1}$. The K_A figure is a measure of the affinity of the cations for the silanol groups. This should be independent of solute but it is obvious from Tables 5.10 and 5.11 that this is not the case. There is also no real trend apparent in the difference between salts. Papp et al.³⁵⁰ reported the use of this equation for the effect of sodium and potassium ions on the retention of aromatic amines. They reported K_A (mmol^{-1}) values of 4.5 and 13.5 for sodium and potassium ions respectively although there was considerable scatter in the data presented. These are about a hundred times greater than the values found here, and no similar difference between the sodium and potassium ions is shown by these figures. The smaller concentrations of ions required to mask the effect of silanols in that paper may be reflective of the packing material; Lichrosorb RP-18 which has a high carbon

Figure 5.19. Fitting Bij's equation to points found using potassium iodide

Conditions: As in Figure 5.6.

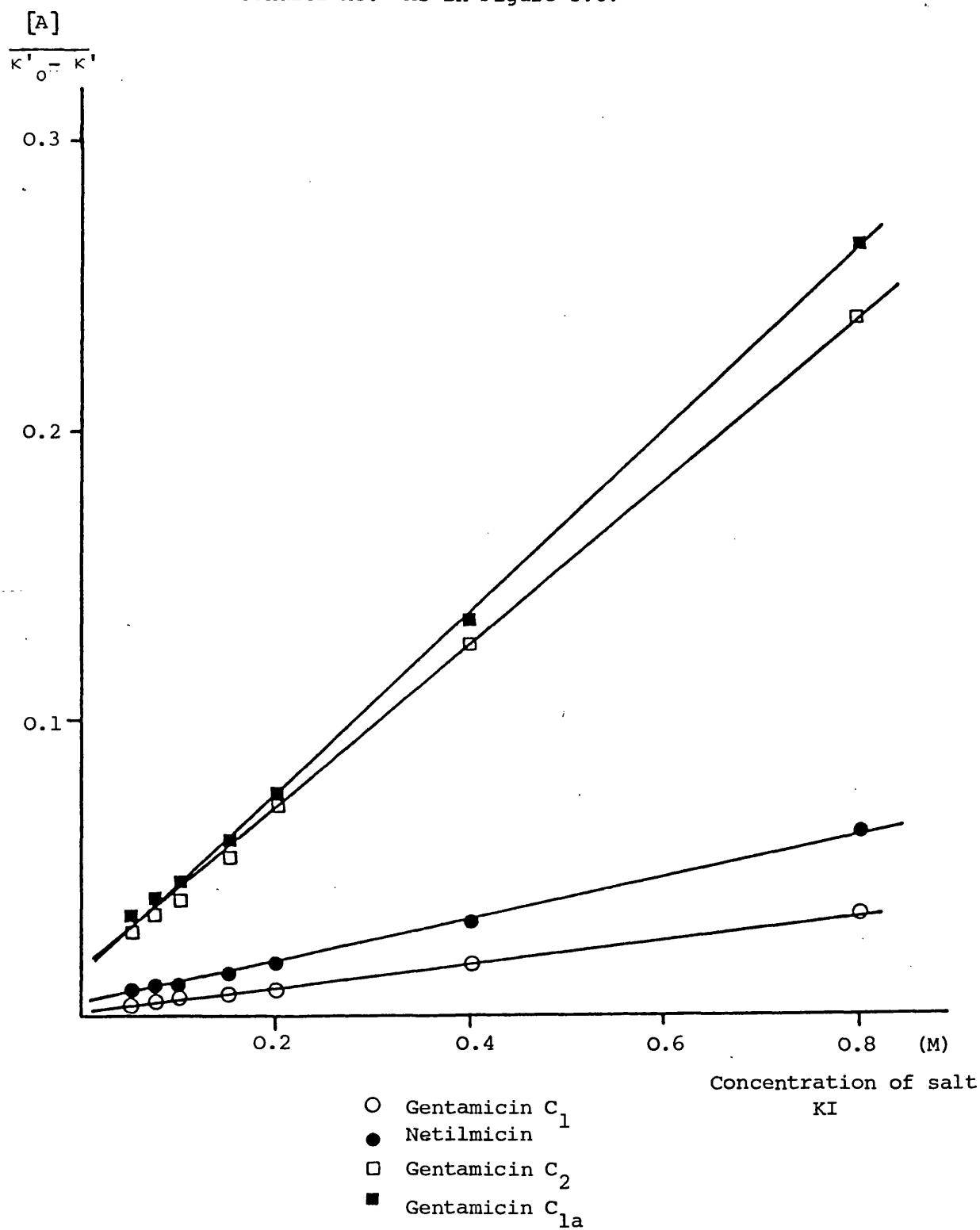


Table 5.10. Fitting Bij's equation to experimental data(Gentamicin C_{1a} and C_2)

Column	Salt	κ_2	K_A (mM ⁻¹)	r	C_A^* (mM)
<u>Gentamicin C_{1a}</u>					
Spherisorb	KI	3.25	0.123	1.000	81.6
"	KSCN	3.37	0.068	0.997	147.9
"	KNO ₃	3.46	0.060	0.999	165.3
"	NaI	3.17	0.133	1.000	75.0
"	NaNO ₃	2.98	0.095	1.000	105.3
"	LiCl	2.72	0.096	0.998	104.5
"	BaCl ₂	3.26	1.852	1.000	5.4
"	EDTA, K ₃	2.59	0.075	0.926	133.0
Zorbax	KI	2.43	0.047	0.995	213.2
"	EDTA, K ₃	2.53	0.011	0.842	892.9
Hypersil	KI	1.65	0.169	0.945	59.2
<u>Gentamicin C_2</u>					
Spherisorb	KI	3.58	0.105	1.000	95.6
"	KSCN	3.79	0.056	0.996	178.4
"	KNO ₃	3.93	0.051	0.996	196.5
"	NaI	3.47	0.119	1.000	84.0
"	NaNO ₃	3.34	0.074	1.000	134.7
"	LiCl	2.94	0.089	0.996	112.9
"	BaCl ₂	3.56	1.890	1.000	5.3
"	EDTA, K ₃	3.09	1.057	0.836	175.7
Zorbax	KI	3.37	0.077	0.997	130.5
"	EDTA, K ₃	3.13	0.028	0.967	358.4
Hypersil	KI	2.24	0.298	0.981	33.6

Table 5.11. Fitting Bij's equation to experimental data

(Gentamicin C_1 and netilmicin)

Column	Salt	κ_2	K_A (mM^{-1})	r	C_A^* (mM)
<u>Gentamicin C_1</u>					
Spherisorb	KI	24.20	0.355	1.000	28.2
"	KSCN	24.49	0.236	1.000	42.4
"	KNO_3	24.47	0.200	1.000	50.1
"	NaI	24.16	0.250	1.000	40.0
"	NaNO_3	24.03	0.177	1.000	56.4
"	LiCl	24.32	0.064	1.000	155.3
"	BaCl_2	24.16	2.381	1.000	4.2
"	EDTA, K_3	21.70	0.215	0.977	46.5
Zorbax	KI	23.87	0.331	1.000	30.5
"	EDTA, K_3	23.52	0.160	0.999	62.4
Hypersil	KI	3.07	0.272	0.973	36.7
<u>Netilmicin</u>					
Spherisorb	KI	14.01	0.135	0.999	73.9
"	KSCN	15.43	0.045	0.980	224.5
"	KNO_3	12.95	0.388	0.983	25.8
"	NaI	12.22	0.248	1.000	40.4
"	NaNO_3	13.62	0.064	1.000	156.6
"	LiCl	12.14	0.036	0.999	280.9
"	BaCl_2	13.55	0.709	1.000	14.1
Zorbax	KI	12.28	0.166	0.999	61.6
"	EDTA, K_3	11.41	0.071	1.000	141.6

loading (22%)³⁰⁶.

The variation in column response dependent on the nature of the solute is illustrated by the C_A^* figures where about a third of the amount of salt is required to make the equivalent amount of silanols for gentamicin C_1 as for the other two C-components. In comparison to the long chain amines used by Bij the C_A^* values are about a hundred times greater in this case. This is as expected since the amines have a hydrophobic chain which can assist penetration of the stationary phase. Bij also noted that amines with hydroxy groups adjacent to the amino function are less effective at masking than the analogue without a polar function. This may arise from a decrease in basicity because of intra-molecular hydrogen bonding. This can be extended to the gentamicins since the secondary amino group at C-3" in the garosamine ring is adjacent to two hydroxy functions. However the secondary amino in the purpurosamine ring is removed from any polar function. On this basis the gentamicin C_1 would be more likely to compete with the salt for the silanol than the other two components.

The κ_2 term can be used to calculate the amount of silanophilic binding present in the retention mechanism. This is achieved by comparing κ_2 to the κ_0 value used in the calculations to give the percentage contribution as shown for differing components and columns in Table 5.12.

Table 5.12. Silanophilic influence on retention

Column	C_{1a}	C_2	C_1	Netilmicin	Calculated silanols
Spherisorb	70.6	67.5	96.8	87.6	70.4
Zorbax	52.8	51.8	95.5	76.7	45.7
Hypersil	36.5	34.1	62.8	-	23.5

The direct comparison of columns and column response to the different nature of solutes is interesting. The gentamicin C_{1a} and C_2 components behave in a similar manner and the extent of silanophilic interaction can be compared with the rough estimations of residual silanols from Table 5.2. It shows a direct relationship and justifies the use of the equation and also the assumption that the silanols are important in the retention mechanism. For the gentamicin C_1 component there is preferential adsorption of the solute by the silanol groups of the Zorbax and Spherisorb columns. Netilmicin is similarly more adsorbed at the silanols but not to such an extent as gentamicin C_1 . An explanation for this effect may be gained by referring to Figure 5.5 and considering the possible conformers. Conformer A is most likely to be predominant in the stationary phase for all components. However, as described before, the gentamicin C_1 component has an additional secondary amino function which may not be masked in this conformation. Thus if ionised it would readily interact with the silanols on the surface. The extra secondary amino group in netilmicin may be partially masked and thus

less able to interact. The presence of a positive charge in this environment will force the equilibrium to an ion-interaction with the silanol. The conformer A for gentamicin C_{1a} and C_2 , which can reduce the influence of the positive charge shows less binding to the silanol groups. The driving force is reduced and hydrogen bonding may be more frequent than ion-exchange. The silanophilic interaction is only proportional to the amount of silanol groups which suggests little kinetic advantage over solvophobic binding. The Hypersil column packing material has fewer available silanols which may restrict the gentamicin C_1 interaction and reduce retention.

The effect of addition of salt to the system is probably one of competition at the silanol groups. The greater the frequency of these groups the higher the salt concentration required to reduce the effect. The greater a solute interacts with the groups, the smaller the concentration of salt needed to reduce retention dramatically. The ion-exchange mechanism is shown to be a stronger retaining force because of the longer retention time of gentamicin C_1 and netilmicin. When the ion-exchange interaction is overcome as at high salt concentration or with small numbers of silanol groups the elution order of gentamicin C_1 then C_{1a} and C_2 , which would be expected by a consideration of the polarity of its derivatives, is found. The Microbondapak C-18 column used for the gentamicin assays³²³ is a similar column to the Hypersil column used here, with 11% carbon loading and endcapping. Thus the 'normal' elution order as expected

was observed with the EDTA salt used. The only exception to the predicted response was in a paper by Marples *et al.*³³³ where a Spherisorb column produced the 'normal' order whereas the reverse would be expected. The salt used was EDTA and at a high concentration, though not high enough to effect a cross-over of components. The only explanation for this is that either the column was more extensively endcapped because of a different batch or a higher ionic strength of the mobile phase was achieved.

Literature Cited

1. Garrod, L.P., Lambert, H.P., O'Grady, F. and Waterworth, P.M. Antibiotic and Chemotherapy, Fifth Edition. Churchill Livingstone, Edinburgh, 1981, 115-154.
2. Kucers, A. and Bennett, N.McK. The Use of Antibiotics, Third Edition. William Heinemann Ltd., London, 1979, 294-416.
3. Schatz, A., Bugie, E. and Waksman, S.A., Proc. Soc. Exp. Biol. Med., 1944, 55, 66-69.
4. Waksman, S.A. and Lechevalier, H.A., Science, 1949, 109, 305-307.
5. Umezawa, H., Ueda, M., Maeda, K., Yagishita, K., Kondō, S., Okami, Y., Utahara, R., Osato, Y., Nitta, K. and Takeuchi, T., J. Antibiot. (Tokyo), Series A, 1957, 10, 181-189.
6. Haskell, T.H., French, J.C., and Bartz, Q.R. J. Amer. Chem. Soc., 1959, 81, 3480-3482.
7. Mori, T., Ichiganagi, T., Kondo, H. and Oda, T. J. Antibiot., 1971, 24, 339-346.
8. Oda, T., Mori, T., Kyotani, Y. and Nakayama, M., J. Antibiot., 1971, 24, 511-518.
9. Weinstein, M.J., Luedemann, G.M., Oden, E.M., Wagman, G.H., Rosselet, J.P., Marquez, J.A., Coniglio, C.T., Charney, W., Herzog, H.L. and Black, J., J. Med. Chem., 1963, 6, 463-464.
10. Weinstein, M.J., Luedemann, G.M., Oden, E.M. and Wagman, G.H. Antimicrobial Agents and Chemotherapy, 1963, 1-7.
11. Weinstein, M.J., Marquez, J.A., Testa, R.T., Wagman, G.H., Oden, E.M. and Waitz, J.A. J. Antibiot., 1970, 23, 551-554.

12. Waksman, S.A. The Actinomycetes. A Summary of Current Knowledge. The Ronald Press Company, New York, 1967, 61-74.
13. Stanier, R.Y., Adelberg, E.A. and Ingraham, J.L. General Microbiology, Fourth Edition, Macmillan Press Ltd., London, 1976, 672-678.
14. Wick, W.E. and Welles, J.S. Antimicrobial Agents and Chemotherapy, 1967, 341-348.
15. Stark, W.M., Hoehn, M.M. and Knox, N.G. Antimicrobial Agents and Chemotherapy, 1967, 314-323.
16. Daniels, P.J.L., Luce, C., Nagabhushan, T.L., Jaret, R.S., Schumacher, D., Reimann, H. and Ilavsky, J., J. Antibiot., 1975, 28, 35-41.
17. Kawaguchi, H., Naito, T., Nakagawa, S. and Fujisawa, K. J. Antibiot., 1972, 25, 695-708.
18. Richardson, K., Jevons, S., Moore, J.W., Ross, B.C. and Wright, J.R. J. Antibiot., 1977, 30, 843-846.
19. Umezawa, H., Umezawa, S., Tsuchiya, T. and Okazaki, Y. J. Antibiot., 1971, 24, 485-487.
20. Wright, J.J., J. Chem. Soc. Chem. Commun., 1976, 206-208.
21. Marquez, J.A. and Kershner, A. in Journal of Chromatography-Volume 15. Antibiotics - Isolation, Separation and Purification. Edited by Weinstein, M.J. and Wagman, G.H. Elsevier Scientific, Amsterdam, 1978, 162-213.
22. Nagabhushan, T.L., Miller, G.H. and Weinstein, M.J. in The Aminoglycosides: Microbiology, Clinical Use and Toxicology Edited by Whelton, A. and Neu, H.C. Marcel Dekker Inc., New York, 1982, 3-28.

23. Brink, N.G., Kuehl, Jr., F.A. and Folkers, K. Science, 1945, 102, 506-507.
24. Kuehl Jr., F.A., Bishop, M.N. and Folkers, K. J. Am. Chem. Soc., 1951, 73, 881-882.
25. Carter, H.E., Dyer, J.R., Shaw, P.D., Rinehart, Jr., K.L. and Hichens, M. J. Am. Chem. Soc., 1961, 83, 372-373.
26. Lemieux, R.U. and Cushley, R.J. Can. J. Chem., 1963, 41, 858-862.
27. Rinehart, Jr., K.L. The Neomycins and Related Antibiotics. John Wiley and Sons Inc., New York, 1964.
28. Chilton, W.S. The Structure of neomycins LP-B and LP-C. Ph.D. Thesis, University of Illinois, 1963.
29. Claes, P.J., Compernelle, F., and Vanderhaeghe, H., J. Antibiot., 1974, 27, 931-942.
30. British Pharmacopeia, 1980. H.M.S.O., London, 1980.
31. Code of Federal Regulations: Food and Drugs 21, Parts 300 to 499. U.S. Government Printing Office, Washington, 1981, Part 444.
32. Decoster, W., Claes, P. and Vanderhaeghe, H. J. Chromatog., 1981, 211, 223-232.
33. Helboe, P. and Kryger, S., J. Chromatog., 1982, 235, 215-220.
34. Tsuji, K., Goetz, J.F., Van Meter, W. and Gusciora, K.A. J. Chromatog., 1979, 175, 141-152.
35. Borowiecka, B. Diss. Pharm. Pharmacol., 1972, 24, 209-215.
36. Chatterjee, N.R. Ind. J. Chem., 1975, 13, 1282-1284.
37. Maehr, H. and Schaffner, C.P. Anal. Chem., 1964, 36(1), 104-108.
38. Majumdar, M.K. and Majumdar, S.K. Appl. Microbiol., 1969, 17, 763-764.

39. Brodasky, T.F. *Anal. Chem.*, 1963, 35, 343-345.
40. Kaiser, D.G. *Anal. Chem.*, 1963, 35, 552-554.
41. Decoster, W., Claes, P. and Vanderhaeghe, J. *Pharm. Sci.*, 1982, 71, 987-991.
42. Inouye, S. and Ogawa, H. *J. Chromatog.*, 1964, 13, 536-541.
43. Borowiecka, B. *Pol. J. Pharmacol. Pharm.*, 1976, 28, 353-359.
44. Mays, D.L., Van Apeldoorn, J. and Lauback, R.G. *J. Chromatog.*, 1976, 120, 93-102.
45. Woo, P.W.K., Dion, W. and Bartz, Q.R. *Tetrahedron Lett.*, 1971, 2625-2628.
46. Price, K.E., Godfrey, J.C. and Kawaguchi, H., in *Structure-Activity Relationships among the Semi-synthetic Antibiotics*. Edited by Perlman, D. Academic Press, New York, 1977, 239-395.
47. Mitsuhashi, S. and Kawabe, H. in *The Aminoglycosides: Microbiology, Clinical Use and Toxicology*. Edited by Whelton, A. and Neu, H.C. Marcel Dekker Inc., New York, 1982, 97-124.
48. Rossi, A., Branca, G., Manzara, S., Morace, G., Polonelli, L., Spagnolo and Ttonci, M. *J. Int. Med. Res.*, 1978, 6, 94-101.
49. Reimann, H., Cooper, D.J., Mallams, A.K., Jaret, R.S., Yehaskel, A., Kugelman, M., Vernay, H.F. and Schumacher, D. *J. Org. Chem.*, 1974, 39, 1451-1457.
50. Moellering, R.C. in *The Aminoglycosides: Microbiology, Clinical Use and Toxicology*. Edited by Whelton, A. and Neu, H.C. Marcel Dekker Inc., New York, 1982, 65-96.
51. Guay, D.R.P. *Drug Intell. Clin. Pharm.*, 1983, 17, 83-91.
52. Cox, D.A., Richardson, K. and Ross, B.C. in *Topics in Antibiotic Chemistry, Volume 1. Aminoglycosides and Ansamycins*. Edited by Sammes, P.G. Ellis Horwood Ltd., England, 1977, 1-90.

53. Rosselet, J.P., Marquez, J., Meseck, E., Murawski, A., Hamden, A., Joyner, C., Schmidt, R., Miglion, D. and Herzog, H.L. Antimicrobial Agents and Chemotherapy-1963, 14-16.
54. Wagman, G.H., Marquez, J.A., Bailey, J.V., Cooper, D., Weinstein, J., Tkach, R. and Daniels, P. J. Chromatog., 1972, 70, 171-173.
55. Wilson, W.L., Richard G. and Hughes, D.W., J. Chromatog., 1973, 78, 442-444.
56. Maehr, H. Gentamicin: Separations and Structural Studies PhD Thesis, Rutgers, The State University, New Brunswick, 1964.
57. Maehr, H. and Schaffner, C.P. J. Chromatog., 1967, 30, 572-578.
58. Nagabhushan, T.L., Turner, W.N., Daniels, P.J.L. and Morton, J.B. J. Org. Chem., 1975, 40, 2830-2834.
59. Nagabhushan, T.L., Daniels, P.J.L., Jaret, R.S. and Morton, J.B. J. Org. Chem., 1975, 40, 2835-2836.
60. Lee, B.K., Condon, R.G., Wagman, G.H. and Katz, E. Antimicrob. Agents Chemother., 1976, 9, 151-159.
61. Lee, B.K., Condon, R.G., Wagman, G.H., Weinstein, M.J. and Katz, E. J. Antibiot., 1976, 29, 677-84.
62. Cooper, D.J. and Yudis, M.D. J. Chem. Soc. Chem. Commun., 1967, 821-822.
63. Cooper, D.J., Marigliano, H.M., Yudis, M.D. and Traubal, T. J. Infect. Dis., 1969, 119, 342-344.
64. Cooper, D.J., Yudis, M.D., Guthrie, R.D. and Prior, A.M. J. Chem. Soc. (C), 1971, 960-962.
65. Cooper, D.J., Yudis, M.D., Marigliano, H.M. and Traubel, T. J. Chem. Soc. (C), 1971, 2876-2879.
66. Cooper, D.J., Daniels, P.J.L., Yudis, M.D., Marigliano, H.M., Guthrie, R.D. and Bukhari, S.T.K., J. Chem. Soc. (C), 1971, 3126-3129.

67. Daniels, P.J.L., Kugelman, M., Mallans, A.K., Tkach, R.W., Vernay, H.F., Weinstein, J. and Yehaskel, A., J. Chem. Soc. Chem. Commun., 1971, 1629-1631.
68. Daniels, P.J.L., Mallans, A.K., Weinstein, J., Wright, J.J. and Milne, G.W.A. J. Chem. Soc. Perkin 1, 1976, 1078-1088.
69. Weinstein, M.J., Wagman, G.H., Oden, E.M. and Marquez, J.A. J. Bacteriol., 1967, 94, 789-790.
70. Wagman, G.H., Marquez, J.A. and Weinstein, M.J. J. Chromatog. 1968, 34, 210-215.
71. Kershner, A.S. Separation, Structural and Physical Studies on the Gentamicin C Complex. PhD Thesis, Rutgers, The State University, New Brunswick, N.J., 1971.
72. Daniels, P.J.L. and Marquez, J.A. United States Patent, 1976, 3,984,395.
73. Byrne, K.M., Kershner, A.S., Maehr, H., Marquez, J.A. and Schaffner, C.P., J. Chromatog., 1977, 131, 191-203.
74. Bérdy, J., Pauncz, J.K., Vajna, Z.M., Horváth, G., Gyimesi, J. and Koczka, I., J. Antibiot., 1977, 30, 945-954.
75. Bérdy, J., Kádár, I., Horváth, G., Méhesfalvi, C., and Gyimesi, J. Sejtosztodas Farmakologiava, 1979, 8, 27-70.
76. Kraisintu, K., Parfitt, R.T. and Rowan, M.G., J. Pharm. Pharmacol., 1980, 72p.
77. Kraisintu, K. Analytical Studies on Gentamicin. PhD Thesis, University of Bath, U.K., 1981.
78. Kraisintu, K., Parfitt, R.T. and Rowan, M.G. Int. J. Pharm., 1982, 10, 67-75.
79. White, L.O. and Reeves, D.S. Proc. 12th ICC Curr. Chemother., 1, 1982, 270-271.

80. Thomas, A.H., J. Pharm. Pharmacol., 1978, 30, 378-379.
81. Calam, D.H., Gilbert, J.N.T., Lightbrown, J.W., Powell, J.W., and Thomas, A.H., J. Pharm. Pharmacol., 1978, 30, 220-232.
82. Freeman, M., Hawkins, P.A., Loran, J.S. and Stead, J.A. J. Liq. Chromatog., 1979, 2, 1305-1317.
83. Thomas, A.H. and Tappin, S.D., J. Chromatog., 1974, 97, 280-283.
84. Wagman, G.H., Bailey, J.V. and Miller, M.M., J. Pharm. Sci., 1968, 57, 1319-1322.
85. Wagman, G.H., Oden, E.M. and Weinstein, M.J. Appl. Microbiol. 1968, 16, 624-627.
86. Wilson, W.L., Richard G. and Hughes, D.W., J. Pharm. Sci., 1973, 62, 282-284.
87. Török, I. and Paál, T.L., J. Chromatog., 1982, 246, 356-359.
88. Kabasakallan, P., Kalliney, S. and Magatti, A.W. Anal. Chem., 1977, 49, 953-955.
89. Anhalt, J.P., Sancilio, J.D. and McCorkle, T., J. Chromatog., 1978, 153, 489-493.
90. Parfitt, R.T., Games, D.E., Rossiter, M., Rogers, M.S. and Weston, A. Biomed. Mass Spect., 1976, 3, 232-234.
91. Jackson, G.G. The Practitioner, 1967, 198, 855-866.
92. De Torres, O.H. Clin. Ther., 1981, 3, 399-412.
93. Jao, R.L. and Jackson, G.G. Antimicrobial Agents and Chemotherapy-1963, 148-152.
94. Noone, P., Pattison, J.R. and Davies, D.G. Postgrad. Med. J. 1974, 50 (Suppl. 7), 9-16.
95. Neu, H.C. in The Aminoglycosides: Microbiology, Clinical Use and Toxicology. Edited by Whelton, A. and Neu, H.C., Marcel Dekker Inc., New York, 1982, 611-628.

96. Franklin, T.J. and Snow, G.A. Biochemistry of Antimicrobial Action, Third Edition, Chapman and Hall, Cambridge, 1981, 118-123.
97. Gale, E.R., Cundliffe, E., Reynolds, P.E., Richmond, M.H. and Waring, M.J. The Molecular Basis of Antibiotic Action, Second Edition. John Wiley and Sons, London, 1981, 419-439.
98. Edwards, D. Antimicrobial Drug Action. The Macmillan Press Ltd., London, 1980, 207-214.
99. Hancock, R.E.W., J. Antimicrob. Chemother., 1981, 8, 249-276.
100. Shannon, K. and Phillips, I., J. Antimicrob. Chemother., 1982, 9, 91-102.
101. O'Grady, F.W. Postgrad. Med. J., 1974, 50, (Suppl. 7), 7-8.
102. Geddes, A.M. in The Control of Antibiotic-Resistant Bacteria. Edited by Stuart-Harris, C.H. and Harris, D.M. Academic Press Inc., London, 1982, 1-16.
103. Phillips, I. and Cooke, D. in The Control of Antibiotic-Resistant Bacteria. Edited by Stuart-Harris, C.H. and Harris, D.M. Academic Press Inc., London, 1982, 201-210.
104. Davies, J., J. Infect. Dis., 1971, 124 (Suppl.), 57-100.
105. Weinstein, M.J., Drube, C.G., Moss Jr., E.L. and Waitz, J.A. J. Infect. Dis., 1971, 124 (Suppl.), 511-17.
106. Reynolds, A.V. and Smith, J.T. in Recent Advances in Infection. Edited by Reeves, D.S. and Geddes, A.M. Churchill Livingstone, Edinburgh, 1979, 165-182.
107. Neu, H.C. in The Aminoglycosides: Microbiology, Clinical Use and Toxicology. Edited by Whelton, A. and Neu, H.C. Marcel Dekker Inc., New York, 1982, 125-142.
108. Rodriguez, V., Stewart, D. and Bodey, G.P. Clin. Pharmacol. Ther., 1969, 11, 275-281.

109. Gingell, J.C. and Waterworth, P.M. Brit. Med. J., 1968, 2, 19-22.
110. Cutler, R.E., Gyselynck, A.-M., Fleet, P. and Forrey, A.W. J.A.M.A., 1972, 219, 1037-1041.
111. Dan, M., Halkin, H. and Rubinstein, E., J. Antimicrob. Chemother., 1981, 7, 551-558.
112. Black, J., Calesnick, B., Williams, D. and Weinstein, M.J. Antimicrobial Agents and Chemotherapy-1963, 138-147.
113. Riff, L.J. and Jackson, G.G., J. Infect. Dis., 1971, 124 (Suppl.), S98-105.
114. Gyselynck, M., Forrey, A. and Cutler, R., J. Infect. Dis., 1971, 124(Suppl.), S70-76.
115. Winters, R.E., Litwack, K.D. and Hewitt, W.L., J. Infect. Dis., 1971, 124 (Suppl.), S90-95.
116. Schumacher, G.E. Am. J. Hosp. Pharm., 1975, 32, 299-308.
117. Walker, J.M., Wise, R. and Mitchard, M., J. Antimicrob. Chemother., 1979, 5, 95-99.
118. Schentag, J.J. in The Aminoglycosides: Microbiology, Clinical Use and Toxicology. Edited by Whelton, A. and Neu, H.C. Marcel Dekker, Inc., New York, 1982, 143-168.
119. Whelton, A. in The Aminoglycosides: Microbiology, Clinical Use and Toxicology. Edited by Whelton, A. and Neu, H.C. Marcel Dekker Inc., New York, 1982, 585-609.
120. McAllister, T.A. Postgrad. Med. J., 1974, 50 (Suppl.), 45-52.
121. Siegel, J.D. and McCracken, G.H. in The Aminoglycosides: Microbiology: Clinical Use and Toxicology. Edited by Whelton, A. and Neu, H.C. Marcel Dekker Inc., New York, 1982, 527-556.

122. Milner, R.D.G. Postgrad. Med. J., 1974, 50 (Suppl.), 40-44.
123. British National Formulary, 1983, Number 5. British Medical Association and the Pharmaceutical Society of Great Britain, London, 1983, 188.
124. Kaka, J.S. and Buchanan, Drug. Intell. Clin. Pharm., 1983, 17, 33-38.
125. Mawer, G.E., Dobbs, S.M., Ahmad, R. and Tooth, J.A. Postgrad. Med. J., 1974, 50 (Suppl.), 31-32.
126. Chan, R.A., Benner, E.J. and Hoeprich, P.P. Ann. Intern. Med., 1972, 76, 773-778.
127. Klastersky, J., Meunier-Carpenter, F. and Prevost, J.M. Am. J. Med. Sci., 1977, 273, 157-167.
128. Bodey, Sr., G.P. in The Aminoglycosides: Microbiology, Clinical Use and Toxicology. Edited by Whelton, A. and Neu, H.C. Marcel Dekker Inc., New York, 1982, 557-583.
129. Andriole, V.T., J. Infect. Dis., 1971, 124 (Suppl.), 546-55.
130. Davies, M., Morgan, J.R. and Anand, C. Antimicrob. Agents Chemother., 1975, 7, 431-434.
131. McLaughlin, J.E. and Reeves, D.S. The Lancet, 1971, 1, 261-264.
132. Kradjan, W.A. and Burger, R. Arch. Intern. Med., 1980, 140, 1668-1670.
133. Tell, S.M., Arwood, L.L. and Visconti, J.A. Am. J. Hosp. Pharm., 1982, 39, 485-486.
134. Ervin, F.R., Bullock, Jr., W.E. and Nuttall, C.E. Antimicrob. Agents Chemother., 1976, 9, 1004-1011.
135. Riff, L.J. and Jackson, G.G. Arch. Intern. Med., 1972, 130, 887-891.

136. Lackner, T.E., Rotschafer, J.C., Crossley, K. and LaBrosse, K.
Am. J. Hosp. Pharm., 1982, 39, 647-648.
137. Lauermann, M.W. and Barza, M. in The Aminoglycosides: Microbiology, Clinical Use and Toxicology. Edited by Whelton, A. and Neu, H.C., Marcel Dekker Inc., New York, 1982, 169-190.
138. Dahlgren, J.G., Anderson, E.T. and Hewitt, W.L. Antimicrob. Agents Chemother., 1975, 8, 58-62.
139. Goodman, E.L., Van Gelder, J., Holmes, R., Hull, A.R. and Sanford, J.P. Antimicrob. Agents Chemother., 1975, 8, 434-438.
140. Jackson, G.G. and Arcieri, G., J. Infect. Dis., 1971, 124 (Suppl.), S130-137.
141. Harper, E.S. and Davey, P.G., J. Antimicrob. Chemother., 1981, 7, 313-317.
142. Whelton, A. in The Aminoglycosides: Microbiology, Clinical Use and Toxicology, Edited by Whelton, A. and Neu, H.C. Marcel Dekker Inc., New York, 1982, 191-222.
143. Schibeci, A. and Schacht, J. Biochem. Pharmacol., 1977, 26, 1769-1774.
144. Gil, D.W., Brown, S.A., Seeholzer, S.H. and Wildey, G.M. Life Sci., 1983, 32, 2043-2046.
145. Baylis, C., J. Antimicrob. Chemother. 1980, 6, 381-388.
146. Soberon, L., Bowman, R.L., Pastoriza-Munoz, E., Kaloyanides, G.J., J. Pharmacol. Exp. Ther., 1979, 210, 334-343.
147. Trollfors, B., Alestig, K., Krantz, I. and Norrby, R. J. Infect. Dis., 1980, 141, 306-309.
148. Hewitt, W.L. Postgrad. Med. J., 1974, 50 (Suppl.), 55-59.
149. Mitchell, C.J., Bullock, S. and Ross, B.D., J. Antimicrob. Chemother., 1977, 3, 593-600.

150. Forrey, A.W., Meijssen-Ludwick, B.T., O'Neill, M.A., Maxwell, B.M., Blair, A.D. and Cutler, R.E. *Toxicol. Appl. Pharmacol.* 1978, 44, 453-62.
151. Lachatre, G., Merle, L., Valette, J.P., Tronchet, J., Charmes, J.P. and Nichot, G. *IRCS Med. Sci. Libr. Compend.* 1982, 10, 857-8.
152. Kohlhepp, S.J., Loveless, M.O., Kohnen, P.W. and Gilbert, D.N. *Abstract of a Conference*, 51.
153. Smith, C.R. and Lietman, P.S. in *The Aminoglycosides: Microbiology, Clinical Use and Toxicology*. Edited by Whelton, A. and Neu, H.C. Marcel Dekker Inc., New York, 1982, 419-452.
154. Bennett, W.M., Plamp, C.E., Gilbert, P.N., Parker, R.A. and Porter, G.A., *J. Infect. Dis.*, 1979, 140, 576-580.
155. Fox, K.E., Brummett, R.E., Brown, R. and Himes, D. *Arch. Otolaryngol*, 1980, 106, 44-49.
156. Brummett, R.E. and Fox, K.E. in *The Aminoglycosides: Microbiology, Clinical Use and Toxicology*. Edited by Whelton, A. and Neu, H.C. Marcel Dekker Inc., New York, 1982, 419-452.
157. Orsulakova, A., Stockhorst, E. and Schact, J., *J. Neurochem.* 1976, 26, 285-290.
158. Anderson, A.C., Hodges, G.R. and Barnes, W.G. *Arch. Intern. Med.*, 1976, 136, 705-707.
159. Waterworth, P.M., *J. Antimicrob. Chemother.*, 1977, 3, 1-8.
160. Wenk, M., *J. Antimicrob. Chemother.*, 1982, 9, 168-169.
161. Follath, F., Wenk, M. and Vozeh, S., *J. Antimicrob. Chemother.* 1981, 8, Suppl. A, 37-43.
162. Maitra, S.K., Yoshikawa, T.T., Guze, L.B. and Schotz, M.C. *Clin. Chem.*, 1979, 25, 1361-1367.

163. Reeves, D.S. Postgrad. Med. J., 1974, 50 (Suppl. 7), 20-21.
164. Bell, S.M. Med. J. Aust., 1976, 2, 481-484.
165. Reeves, D.S. and Bywater, M.J., J. Antimicrob. Chemother., 1975, 1, 103-116.
166. Fabius, G.T.J. and Mouton, R.P. in Chemotherapy, Vol. 2. Edited by Williams, J.D. and Geddes, A.M. Plenum Press, New York, 1976, 47-54.
167. Jarvis, J.D. and Leung, T.W.C. in Chemotherapy, Vol. 2. Edited by Williams, J.D. and Geddes, A.M., Plenum Press, New York, 1976, 143-145.
168. Sherris, J.C. in Chemotherapy Vol. 2. Edited by Williams, J.D. and Geddes, A.M., Plenum Press, New York, 1976, 1-6.
169. Stessman, J., Michel, J. and Sacks, T. Chemotherapy (Basel), 1977, 239, 142-148.
170. Shanson, D.C., Hince, C.J. and Daniels, J.V. in Chemotherapy, Vol. 2, Edited by Williams, J.D. and Geddes, A.M., Plenum Press, New York, 1976, 147-153.
171. Shanson, D.C. and Hince, C.J., J. Antimicrob. Chemother., 1977, 3, 563-570.
172. Shanson, D.C. and Hince, C., J. Clin. Path., 1977, 30, 521-525.
173. Giamarellou, H., Zimelis, V.M., Matulionis, D.O. and Jackson, G.G., J. Infect. Dis., 1975, 132, 399-406.
174. Reeves, D.S. and Holt, H.A. in Chemotherapy, Vol. 2, Edited by Williams, J.D. and Geddes, A.M., Plenum Press, New York, 1976, 133-141.
175. Andrews, J., Gillette, P., Williams, J.D. and Mitchard, M. Postgrad. Med. J., 1974, 50 (Suppl. 7), 17-20.
176. Staneck, J.L., Land, G.A., Cordes, V., Glenn, S. and

- Resendes, J.P. Clin. Biochem. Anal., 1981, 10, 171-181.
177. Männistö, P.T. Methods Find. Exp. Clin. Pharmacol., 1982, 4, 167-171.
 178. Hospes, W., Boskma, R.J. and Brouwers, J.R.B.J. Pharm. Week. Bl. Sci. Ed., 1982, 4, 32-37.
 179. Delaney, C.J., Opheim, K.E., Smith, A.L. and Plorde, J.J. Antimicrob. Agents Chemother., 1982, 21, 19-25.
 180. Stobberingh, E.E., Houben, A.W., Schwillens, P.L.W.J. and Van Boven, C.P.A. Antonie van Leeuwenhoek, 1981, 47, 80-81.
 181. Acton, W.J., Van Duyn, O.M., Allen, L.V. and Flournoy, D.J. Clin. Chem., 1982, 28, 177-180.
 182. Ratcliff, R.M., Mirelli, C., Moran, E., O'Leary, D. and White, R. Antimicrob. Agent. Chemother., 1981, 19, 508-512.
 183. Reeves, D.S., J. Antimicrob. Chemother., 1981, 8, Suppl. A, 51-54.
 184. Qadri, S.M.H., Gillum, R.L. and Boyd, D. Antonie van Leeuwenhoek, 1982, 48, 209-218.
 185. Johnson, J.E., Crawford, S. and Jorgensen, J.H., J. Clin. Microbiol., 1982, 16, 299-302.
 186. Smith, D.H., Van Otto, B. and Smith, A.L. New Eng. J. Med., 1972, 286, 583-586.
 187. Phillips, I., Warren, C. and Smith, S.E., J. Clin. Path., 1974, 27, 447-451.
 188. Broughall, J.M. and Reeves, D.S. in Chemotherapy, Vol. 2. Edited by Williams, J.D. and Geddes, A.M. Plenum Press, New York, 1976, 159-163.
 189. Case, R.V. and Mezei, L.M. Clin. Chem., 1978, 24, 2145-2150.
 190. Shannon, K.P. and Phillips, I., J. Antimicrob. Chemother., 1977, 3, 25-33.

191. Daigneault, R., Gagné, M. and Brazeau, M., J. Infect. Dis., 1974, 130, 642-645.
192. Matzke, G.R., Gwizdala, C., Wery, J., Ferry, D., and Starnes, R. Ther. Drug. Monit., 1982, 4, 195-200.
193. Bolton, A.E. and Hunter, W.M. Biochem. J., 1973, 133, 529-539.
194. Jonsson, S. in Chemotherapy, Vol. 2. Edited by Williams, J.D. and Geddes, A.M. Plenum Press, New York, 1976, 165-168.
195. Watson, R.A.A., Shaw, E.J. and Edwards, C.R.W. in Chemotherapy, Vol. 2. Edited by Williams, J.D. and Geddes, A.M. Plenum Press, New York, 1976, 107-110.
196. Ashby, C.D., Lewis, J.E. and Nelson, J.C. Clin. Chem., 1978, 24, 1734-1737.
197. Broughton, A. and Strong, J.E. Clin. Chim. Acta, 1976, 66, 125-129.
198. Matzke, G.R., Hunt, S., Gwizdala, C. and Cronk, J.D. Am. J. Hosp. Pharm., 1980, 37, 1049-1050.
199. Oeltgen, P.R., Hamann, S.R. and Blouin, R.A. Ther. Drug Monit., 1980, 2, 423-425.
200. Standefer, J.C. and Saunders, G.C. Clin. Chem., 1978, 24, 1903-1907.
201. Mattiasson, B., Svensson, K., Borrebaeck, C., Jonsson, S. and Kronvall, G. Clin. Chem., 1978, 24, 1770-1773.
202. Hooymans, P.M., Cilissen, J. and Merkus, F.W.H.M. Am. J. Hosp. Pharm., 1982, 39, 784, 787 and 788.
203. Pesce, M.A. and Bodourian, S.H. Clin. Chem., 1981, 27, 1460-1462.
204. Cox, C. and Buret, J. Anal. Chem., 1982, 54, 1862-1865.
205. Singh, P., Leung, D.K., Rowley, G.L., Gagne, C. and Ullman, E.F. Anal. Biochem., 1980, 104, 51-58.

206. Emit-aed Instruction Book. Syva, California, 1978.
207. Carlson, L.G., Delaney, C.J. and Plorde, J.J. Antimicrob. Agents Chemother., 1982, 21, 192-194.
208. Watson, R.A.A., London, J., Shaw, E.J. and Smith, D.S. Clin. Chim. Acta, 1976, 73, 51-55.
209. Shaw, E.J., Watson, R.A.A., London, J. and Smith, D.S., J. Clin. Path., 1977, 30, 526-531.
210. Burd, J.F., Wong, R.C., Feeney, J.E., Carrico, R.J. and Boguslaski, R.C.; Clin. Chem., 1977, 23, 1402-1408.
211. Burd, J.F. Method Enzymol., 1981, 74, 79-87.
212. Jolley, M.E., Stroupe, S.D., Wang, C.H.J., Panas, H.N., Keegan, C.L., Schmidt, R.L. and Schwenzer, K.S. Clin. Chem., 1981, 27, 1190-1197.
213. Munro, A.J., Smith, D.S. and Shaw, E.J., J. Antimicrob. Chemother., 1982, 9, 47-51.
214. Dandliker, W.B., Hsu, M.L., Levin, J. and Rao, B.R. Method Enzymol., 1981, 74, 3-27.
215. Meffin, P.J. and Miners, J.O. in Progress in Drug Metabolism Volume 4. Edited by Bridges, J.W. and Chasseaud, L.F. John Wiley and Sons, New York, 1980, 261-308.
216. Anhalt, J.P. Chromatog. Sci., 1979, 12 (Biol./Biomed 2), 1-16.
217. Reid, E. Analyst, 1976, 101, 1-18.
218. Nilsson-Ehle, I. Chromatog.Sci., 1982, 20, 173-184.
219. Mayhew, J.W. and Gorbach, S.L., J. Chromatog., 1978, 151, 133-146.
220. Peng, G.W., Gadalla, M.A.F., Peng, A., Smith, V. and Chiou, W.L. Clin. Chem., 1977, 23, 1838-1844.
221. Peng, G.W., Jackson, G.G. and Chiou, W.L. Antimicrob. Agents

- Chemother., 1977, 12, 707-709.
222. Chiou, W.L., Nation, R.L, Peng, G.W. and Huang, S.M. Clin. Chem., 1978, 24, 1846-1847.
223. Maitra, S.K., Yoshikawa, T.T., Hansen, J.L., Nilsson-Ehle, I., Palin, W.J., Schotz, M.C. and Guze, L.B. Clin. Chem., 1977, 23, 2275-2278.
224. Maitra, S.K., Yoshikawa, T.T., Hansen, J.L., Schotz, M.C. and Guze, L.B. Am. J. Clin. Pathol., 1979, 71, 428-432.
225. Anhalt, J.P. and Brown, S.D. Clin. Chem., 1978, 24, 1940-1947.
226. Anhalt, J.P. Antimicrob. Agents Chemother., 1977, 11, 651-655.
227. Kubo, H., Kinoshita, T., Kobayashi, Y. and Tokunaga, K. J. Chromatog., 1982, 227, 244-248.
228. Barends, D.M., Van der Sandt, J.S.F. and Hulshoff, A., J. Chromatog., 1980, 182, 201-210.
229. Larsen, N.E., Marinelli, K. and Heilesen, A.M., J. Chromatog., 1980, 221, 182-187.
230. Krugers Dagneaux, P.G.L.C. and Klein Elhorst, J.T. Pharm. Week. Bl. Sci. Ed., 1981, 3, 66-70.
231. Bäck, S.E., Nilsson-Ehle, I. and Nilsson-Ehle, P. Clin. Chem., 1979, 25, 1222-1225.
232. Haughey, D.B., Janicke, D.M., Adelman, M. and Schentag, J.J. Antimicrob. Agents Chemother., 1980, 17, 649-653.
233. Marples, J. and Oates, M.D.G., J. Antimicrob. Chemother., 1982, 9, 311-318.
234. D'Souza, J. and Ogilvie, R.I., J. Chromatog., 1982, 232, 212-218.
235. Walker, S.E. and Coates, P.E., J. Chromatog., 1981, 223, 131-138.
236. Frei, R.W. and Lawrence, J.F. Chemical Derivatisation in

Analytical Chemistry, Volume 1: Chromatography. Plenum Press, New York, 1981, 127-196.

237. Roth, M. Anal. Chem., 1971, 43, 880-882.
238. Lawrence, J.F., J. Chromatog. Sci., 1979, 17, 147-151.
239. Bristow, P.A. Liquid Chromatography in Practise. hetp, Cheshire, 1976, 179 and 187.
240. Frei, R.W., J. Chromatog., 1979, 165, 75-86.
241. Snyder, L.R. and Kirkland, J.J. Introduction to Modern Liquid Chromatography, Second Edition. John Wiley and Sons, New York, 1979, 16-82.
242. Jones, S.M., Blazevic, D.J. and Balfour, Jr., H.H. Antimicrob . Agents Chemother., 1976, 10, 866-867.
243. Faber, D.B., MacLaren, D.M. and De Goede, P.N.F.C. Proc. 12th ICC Curr. Chemo. Immunotherapy, 1982, 2, 800-802.
244. Reeves, R.E. Adv. Carbohydrate Chem., 1951, 6, 107-134.
245. Maehr, H. and Schaffner, C.P., J. Am. Chem. Soc., 1967, 89, 6787-6788.
246. Morton, J.B., Long, R.C., Daniels, P.J.L., Tkach, R.W. and Goldstein, J.H., J. Am. Chem. Soc., 1973, 95, 7464-7469.
247. Naito, T., Toda, S., Nakagawa, S. and Kawaguchi, H. ACS Symp. Ser., 1980, 125, 257-294.
248. Burlingame, A.L., Dell, A. and Russell, D.H. Anal. Chem., 1982, 54, 363R-409R.
249. Howe, I., Williams, D.H. and Bowen, R.D. Mass Spectrometry, Principles and Applications, Second Edition, McGraw-Hill, Inc., New York, 1981, 4-49.
250. Daves, G.D. Acc. Chem. Res., 1979, 12, 359-365.
251. Beckey, H.D. Int. J. Mass Spectrom. Ion Phys., 1969, 2, 500-503.

- 252. Merritt, C. and McEwan, C.N. Mass Spectrometry , Part A, Volume 3. Marcel Dekker Inc., New York, 1979, 145-266.
- 253. Holland, J.F., Soltmann, B. and Sweeley, C.C. Biomed. Mass Spectrom. 1976, 3, 340-345.
- 254. McNeal, C.J. Anal. Chem., 1982, 54, 44A-50A.
- 255. Dell, A., Morris, H.R., Levin, M.D. and Hecht, S.M. Biochem. Biophys. Res. Commun., 1981, 102, 730-738.
- 256. Dell, A., Hider, R.C., Barber, M., Bordoli, R.S., Sedgwick, R.D., Tyler, A.N. and Neilands, J.B. Biomed. Mass Spectrom., 1982, 9, 159-161.
- 257. Williams, D.H., Bradley, C., Bojesen, G., Santikarn, S. and Taylor, L.C.E., J. Am. Chem. Soc., 1981, 103, 5700-5704.
- 258. Fenselau, C. Anal. Chem., 1982, 54, 105A-116A.
- 259. Macfarlane, R.D. and Torgerson, D.F. Science, 1976, 191, 920-925.
- 260. Stimpson, B.P. and Evans, C.A. Biomed. Mass Spectrom., 1978, 5, 52-63.
- 261. Lai, S.T.F. and Evans, C.T.A. Biomed. Mass Spectrom., 1979, 6, 10-14.
- 262. Lai, S.T.F., Chan, K.W. and Cook, K.W. Macromolecules, 1980, 13, 953-959.
- 263. Posthumus, M.A., Kistemaker, P.G., Meuzelaar, and Ten Noever de Brauw, M.C. Anal. Chem., 1978, 50, 985-991.
- 264. Day, R.J., Unger, S.E. and Cooks, R.G. Anal. Chem., 1980, 52, 557A-572A.
- 265. Garrison, B.J. and Winograd, N. Science, 1982, 216, 805-812.
- 266. Aberth, W., Straub, K.M. and Burlingame, A.L. Anal. Chem., 1982, 54, 2029-2034.
- 267. Surman, D.J. and Vickerman, J.C., J. Chem. Soc. Chem. Commun.,

- 1981, 324-325.
268. Barber, M., Bordoli, R.S., Sedgwick, R.D. and Tyler, A.N.
J. Chem. Soc. Chem. Commun., 1981, 325-327.
269. Barber, M., Bordoli, R.S., Sedgwick, R.D. and Tyler, A.N.
Nature, 1981, 293, 270-275.
270. Surman, D.J. and Vickerman, J.C., J. Chem. Res. (S), 1981,
170-171.
271. Martin, S.A., Costello, C.E. and Blemann, K. Anal. Chem.,
1982, 54, 2362-2368.
272. Rinehart, Jr., K.L., Gaudioso, L.A., Moore, M.L., Pandey,
R.C., Cook, J.C., Barber, M., Sedgwick, R.D., Bordoli, R.S.,
Tyler, A.N. and Green, B.N., J. Am. Chem. Soc., 1981, 103,
6517-6520.
273. Levy, G.C. and Nelson, G.L. Carbon-13 Nuclear Magnetic
Resonance for Organic Chemists. John Wiley and Sons, New
York, 1972, 38-49.
274. Bremser, W., Ernst, L., Franke, B., Gerhards, R. and
Hardt, A. Carbon-13 NMR Spectral Data. Verlag Chemie,
Weinheim, 1981.
275. Billman, J.H. and McDowell, J.W., J. Org. Chem., 1961, 26,
1437-1440.
276. Norman, R.O.C. Principles of Organic Synthesis, Second Edition,
Chapman and Hall, London, 1978, 649.
277. Cragg, G.M.L. Organoboranes in Organic Synthesis. Marcel
Dekker Inc., New York, 1973, 319-354.
278. Bellamy, L.J. The Infra-Red Spectra of Complex Molecules,
Third Edition, Chapman and Hall, London, 1975, 294-303.
279. Davis, T.P., Gehrke, C.W., Gehrke, Jr., C.W., Cunningham, T.D.,

- Kuo, K.C., Gerhardt, K.O., Johnson, H.D. and Williams, C.H.
J. Chromatog., 1979, 162, 293-310.
280. Buteau, C., Duitschaeffer, C.L. and Ashton, G.C., J. Chromatog.
1981, 212, 23-27.
281. Skadden, T. and Greibrokk, T., J. Chromatog., 1982, 247, 111-122.
282. Hill, D.W., Walters, F.H., Wilson, T.D. and Stuart, J.D. Anal.
Chem., 1979, 51, 1338-1341.
283. Carroll, S.F. and Nelson, D.R. Anal. Biochem., 1979, 98, 190-197.
284. Larsen, B.R. and West, F.G., J. Chromatog. Sci., 1981, 19,
259-265.
285. Lindroth, P. and Mopper, K. Anal. Chem., 1979, 51, 1667-1674.
286. Simons, Jr., S.S. and Johnson, D.F., Anal. Biochem., 1978,
90, 705-725.
287. Simons, Jr., S.S. and Johnson, D.F. Anal. Biochem., 1977,
82, 250-254.
288. Simons, Jr., S.S. and Johnson, D.F., J. Chem. Soc. Chem. Commun.,
1977, 374-375.
289. Simons, Jr., S.S. and Johnson, D.F., J. Org. Chem., 1978,
43, 2886-2891.
290. Nakamura, H., Matsumoto, A. and Tamura, Z. Anal. Lett.,
1982, 15 (A17), 1393-1410.
291. Simons, Jr., S.S. and Johnson, D.F., J. Am. Chem. Soc., 1976,
98, 7098-7099.
292. Bonnett, R. and Brown, R.F.C., J. Chem. Soc. Chem. Commun.,
1972, 393-395.
293. Bornstein, J., Remy, D.E. and Shields, J.E. Tetrahedron
Lett., 1974, 48, 4247-4250.
294. Palmer, M.H. and Kennedy, S.M.F., J. Chem. Soc. Perkin II,
1976, 81-89.

295. Black, P.J., Brown, R.D. and Heffernan, M.L. Aust. J. Chem., 1967, 20, 1305-1323.
296. Remers, W.A. and Brown, R.K. The Chemistry of Heterocyclic Compounds: Indoles, Part One. Edited by Houlihan, W.J. John Wiley and Sons New York, 1972, 48-49.
297. Pouchert, C.J. and Campbell, J.R. The Aldrich Library of NMR Spectra, Volume VI, Aldrich Chemical Company Inc., 1974, 81.
298. Begtrup, M., Claramunt, R.M. and Elguero, J., J. Chem. Soc. Perkin II, 1978, 99-104.
299. Lowry, T.H. and Richardson, K.S. Mechanism and Theory in Organic Chemistry, Second Edition. Harper and Row, New York, 1981, 625-640.
300. Majors, R.E., Barth, H.G. and Lochmüller, C.H. Anal. Chem., 1982, 54, 323R-363R.
301. Halász, I. Anal. Chem., 1980, 52, 1393A-1403A.
302. Colin, H. and Guiochon, G., J. Chromatog., 1977, 141, 289-312.
303. Unger, K.K. Journal of Chromatography Library, Vol. 16; Porous Silica, Its Properties and Uses as Support in Column Liquid Chromatography. Elsevier, Amsterdam, 1979.
304. Karch, K., Sebastian, I. and Halász, I., J. Chromatog., 1976, 122, 3-16.
305. Cox, G.B., J. Chromatog. Sci., 1977, 15, 385-392.
306. Majors, R.E., J. Chromatog. Sci., 1980, 18, 488-511.
307. Horváth, C. Trends Anal. Chem. ERS Ed., 1981, 1, 6-12.
308. Cooke, N.H.C. and Olsen, K., J. Chromatog. Sci., 1980, 18, 512-524.
309. Iler, R.K., J. Chromatog., 1981, 209, 341-342.
310. Bush, S.G., Jorgenson, J.W., Miller, M.L. and Linton, R.W.

- J. Chromatog., 1983, 260, 1-12.
311. Melander, W., Stoveken, J. and Horváth, C., J. Chromatog., 1980, 199, 35-56.
312. Horváth, C. and Melander, W., J. Chromatog. Sci., 1977, 15, 393-404.
313. Nahum, A. and Horváth, C., J. Chromatog., 1981, 203, 53-63.
314. Tanaka, N., Goodell, H. and Karger, B.L., J. Chromatog., 1978, 158, 233-248.
315. Colin, H. and Guiochon, G., J. Chromatog., 1978, 158, 183-205.
316. Melander, W., Chen, B.K. and Horváth, C., J. Chromatog., 1979, 185, 99-109.
317. Riley, C.M., Tomlinson, E. and Jefferies, T.M., J. Chromatog., 1979, 185, 197-224.
318. Tomlinson, E., Poppe, H. and Kraak, J.C. Int. J. Pharm., 1981, 7, 225-243.
319. Karger, B.L., Snyder, L.R. and Horváth, C. An Introduction to Separation Science. John Wiley and Sons, New York, 1973, 12-61.
320. Locke, D.C., J. Chromatog. Sci., 1974, 12, 433-437.
321. Horváth, C., Melander, W. and Molnár, I. Anal. Chem., 1977, 49, 142-154.
322. Zakaria, M. and Brown, P.R., J. Chromatog., 1983, 255, 151-162.
323. Zakaria, M., Brown, P.R. and Grushka, E. Anal. Chem., 1983, 55, 457-463.
324. Hansson, C., Agrup, G., Rorsman, H., Rosengren, A.M., Rosengren, E. and Edholm, L.E., J. Chromatog., 1979, 162, 7-22.
325. Rudzinski, W.E., Bennett, D., Garcia, V. and Seymour, M. J. Chromatog. Sci., 1983, 21, 57-61.

326. Van de Venne, J.L.M., Hendrikz and Deelder, R.S., J. Chromatog., 1978, 167, 1-16.
327. Sokdowski, A. and Wahlund, K.G., J. Chromatog., 1980, 189, 299-316.
328. Eksborg, S. and Ekqvist, B., J. Chromatog., 1981, 209, 161-173.
329. Varga-Puchony, Z. and Vigh, G., J. Chromatog., 1983, 257, 380-383.
330. Bij, K.E., Horváth, C., Melander, W.R. and Nahum, A. J. Chromatog., 1981, 203, 65-84.
331. Tilly Melin, A., Ljungcrantz, M. and Schill, G., J. Chromatog., 1979, 185, 225-239.
332. Van der Houwen, O.A.G.J., Sorel, R.H.A., Hulshoff, A., Teeuwsen J. and Indemans, A.W.M., J. Chromatog., 1981, 209, 393-404.
333. Horváth, C., Melander, W., Molnar, I. and Molnar, P. Anal. Chem., 1977, 49, 2295-2305.
334. Bidlingmeyer, B.A., J. Chromatog., Sci., 1980, 18, 525-539.
335. Tilly Melin, A., Askemark, Y., Wahlund, K-G, and Schill, G. Anal. Chem., 1979, 51, 976-983.
336. Greving, J.E., Bouman, H., Jonkman, J.H.G., Westenberg, H.G.M. and De Zeeuw, R.A., J. Chromatog., 1979, 186, 683-690.
337. Bidlingmeyer, B.A. and Warren, Jr., F.V. Anal. Chem., 1982, 54, 2351-2356.
338. Bidlingmeyer, B.A., Deming, S.N., Price, Jr., W.P., Sachok, B. and Petrusek, M., J. Chromatog., 1979, 186, 419-434.
339. Knox, J.H. and Jurand, J., J. Chromatog., 1976, 125, 89-101.
340. Hoffman, N.E. and Llao. Anal. Chem., 1977, 49, 2231-2234.
341. Hancock, W.S., Bishop, C.A., Buttersby, J.E., Harding, D.R.K. and Hearn, M.T.W., J. Chromatog., 1979, 168, 377-384.

- 342. Kraak, J.C., Jonker, K.M. and Huber, J.F.K., J. Chromatog., 1977, 142, 671-688.
- 343. Kissinger, P.T. Anal.Chem., 1977, 49, 883.
- 344. Bidlingmeyer, B.A., Del Rios, J.K. and Korpl, J. Anal. Chem., 1982, 54, 442-447.
- 345. Wheals, B.B., J. Chromatog., 1980, 187, 65-85.
- 346. Zhelyazkov, D., Ivanova, K., Gueorguiev, N., Marinova, R., Beltcheva, A., Mangurova, M. and Temnyalov, N. in Chemotherapy, Vol. 2. Edited by Williams, J.D. and Geddes, A.M. Plenum Press, New York, 1976, 409-415.
- 347. Thurman, E.M., J. Chromatog., 1979, 185, 625-634.
- 348. Nelson, J.J., J. Chromatog., Sci., 1973, 11, 28-35.
- 349. Benson, Jr., J.V., Gordon, M.J. and Patterson, J.A. Anal. Biochem., 1967, 18, 228-240.
- 350. Papp, E. and Vigh, G. J. Chromatog., 1983, 259, 49-58.
- 351. Stranahan, J.J. and Deming, S.N. Anal. Chem., 1982, 54, 2251-2256.
- 352. McDonald, R.S. and Martin, E.V. Can. J. Chem., 1979, 57, 506-516.
- 353. DoMinh, T., Johnson, A.L., Jones, J.E. and Senise, P.P. J. Org. Chem., 1977, 42, 4217-4221.
- 354. Drug Treatment, 2nd Edition. Edited by Avery, G.S. Churchill Livingstone, London, 1980, 1214.